INFLUENCE OF DIETARY FATS ON CARCINOGENESIS

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by

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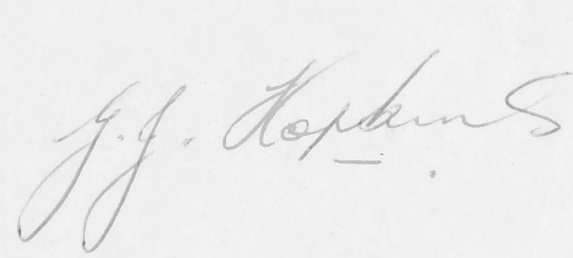
This thesis is my own work. Any collaboration with colleagues is acknowledged in the text.



G.J. Hopkins

TABLE OF CONTENTS

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ABSTRACT

The effect of different dietary fats on chemical-induced carcinogenesis was examined by feeding animals diets containing either sunflower-seed oil (polyunsaturated fat diet) or tallow (saturated fat diet). In addition, the changes induced by these diets in the structure and function of several biological systems involved in carcinogenesis were studied.

1. The microsomal mixed function oxygenase system, which contains the haemoprotein cytochrome P-450, is responsible for the metabolism of many chemical carcinogens. Rats fed the saturated fat diet had greater rates of drug metabolism, as assessed by barbiturate-induced sleeping times, and greater concentrations of hepatic microsomal cytochrome P-450 than did rats fed the polyunsaturated fat diet. Similar effects of the diets on the concentration of cytochrome P-450 were observed when microsomal metabolism was stimulated by the administration of pentobarbitone to the rats. The results of experiments in other laboratories indicate that these changes in metabolism would have enhanced the susceptibility of rats fed the polyunsaturated fat diet to the chemical-induction of tumours.

2. The influence of the polyunsaturated and saturated fat diets on chemical carcinogenesis was examined in rats dosed with the carcinogenic polycyclic hydrocarbon, 7,12-dimethylbenz(a)anthracene (DMBA). A greater tumorigenic

response occurred in rats fed the polyunsaturated fat diet than in rats fed the saturated fat diet. Since only the diet fed after DMBA administration influenced the tumour incidence, the enhancement of carcinogenesis probably involved the survival and proliferation of neoplastic cells and not the initial event of neoplastic transformation. This indicates that the diet-induced changes in the mixed function oxygenase system (section 1), were not important in determining the tumorigenic response of the DMBA-dosed rats.

3. The effect of the dietary fats on DMBA-induced carcinogenesis in C3HA^{vy}fB mice was examined. An amount of DMBA which would induce tumours in these mice was determined and given to mice fed the polyunsaturated and saturated fat diets. A greater tumorigenic response occurred in mice fed the polyunsaturated fat diet compared to mice fed the saturated fat diet. As with the DMBA-dosed rats, only the diet fed after DMBA administration influenced the tumour incidence.

4. Since the enhancement of chemical-induced carcinogenesis by the polyunsaturated fat diet appeared to be exerted during the promotional stage of carcinogenesis, the effect of the diets on the proliferation of tumour cells was examined. The number of mice which developed tumours after inoculation with a suspension of single cells from a transplantable mammary adenocarcinoma was greater when mice were fed the polyunsaturated fat diet rather than the saturated fat diet.

5. Purified hepatocyte plasma membranes were prepared from mice fed the same dietary regimes as were given to the DMBA-dosed animals. When mice were fed the polyunsaturated fat diet the proportion of polyunsaturated fatty acids in the hepatocyte plasma membrane lipids increased, reaching a maximum after 4 weeks and returning to lower levels with continued feeding of the diet. The existence of this transient increase in unsaturation was confirmed in a second experiment. When mice were fed the saturated fat diet the proportion of the major dietary unsaturated fatty acid, oleic acid, increased. As changes in fatty acid composition alter membrane function, this transient increase in unsaturation of the plasma membrane lipids of mice fed the polyunsaturated fat diet may have been important in determining the enhanced tumorigenic response of the animals fed this diet after dosing with DMBA.

PUBLICATIONS

Some of the work presented in this thesis has been published:

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INTRODUCTION

Carcinogenesis refers to the causative factors in tumour induction and their modes of action. Ancillary factors which modify the action of carcinogenic agents or alter the progression of the disease are also involved. Substantial progress in the clinical and experimental study of these aspects of cancer has been made during the past two or three decades. Today, a clear distinction can be made between mortality and morbidity due to cancer. Nevertheless, the number of deaths due to most types of cancer is increasing and this increase cannot be explained on the basis of an increase in population or a change in the age structure of the population (Süss *et al.*, 1973). These facts point to the conclusion that the increase in deaths due to cancer may be due to an increasing exposure to causative factors.

It was once thought that only narrowly circumscribed occupational groups came in contact with carcinogenic agents (Süss *et al.*, 1973; Berenblum, 1974). It is now evident, however, that much of our environment is contaminated with substances which induce cancer or may potentiate the development of cancer. Such contaminants are thought to be wholly or partly responsible for the majority of cancers seen in man (Maugh, 1974). Cancer prevention can be achieved in two ways; by eliminating the carcinogenic or potentiating factors from the environment, or by preventing the full realisation of the carcinogenic action after carcinogenesis has been initiated. Since present knowledge of the causation of cancer is more

advanced than knowledge of the specific disturbances in the cancer tissue it is likely that greater benefit will be gained, in the near future anyway, from cancer prevention than from a cancer cure. On purely theoretical grounds, the eradication of carcinogenic and cancer-potentiating agents from our environment would provide a highly significant relief from the burden of cancer. However, achievement of this aim is unlikely because the task of assaying all suspected substances for safety is monumental. As the carcinogenicity of a substance is usually determined from experiments with animals, which may not be applicable to man, the risk-benefit ratio to society may be an important factor. It may also be very difficult to stop or decrease exposure to potentially harmful agents which are, for example, essential dietary constituents or are irreversibly associated with an essential dietary constituent.

In the present review the effect of one environmental factor, namely dietary fat, on carcinogenesis is examined. The results of experiments from several different fields of research are discussed in relation to the possible mechanisms by which dietary fats may influence carcinogenesis. In addition, several potentially fruitful areas for further investigation are suggested.

1. Epidemiological studies on humans

Epidemiological data on humans has shown that the

incidence of certain types of cancer varies between countries (Segi *et al*., 1969). Lea (1965, 1966a) examined the mortality from breast cancer and noted a correlation with geographical latitude and subsequently with environmental temperature. Since diet will be influenced to some extent by environmental temperature, Lea (1966b) examined the consumption of dietary constituents in different countries and found a highly significant correlation between the consumption of fats and oils and mortality from breast cancer. The consumption of fats and oils was also correlated with death-rates from cancers of the ovaries, prostate, pancreas, bladder, intestine and rectum and with death-rates from Hodgkin's disease, lymphosarcoma and leukaemia in people over 55 years of age (Lea, 1966b, 1967). On the other hand, death-rates from cancers of the liver, stomach and uterus were negatively correlated with the consumption of fats and oils (Lea, 1967).

During extensive analysis of epidemiological data on humans, Wynder *et al*. (1967) noted a significant correlation between mortality from cancer of the colon and mortality from breast cancer in women from different countries. Dietary factors, such as the consumption of fats, or related socio-economic variables may have influenced the incidence of these tumours. It was suggested that the increased consumption of fats could have enhanced the risk of breast cancer by altering the production of hormones or by prolonging the retention of hormones in the adipose tissue (Wynder, 1968,

1969). Hems (1970) has also suggested that the influence of dietary fat consumption on breast cancer is more important late in life. The suggestion of Wynder *et al.* (1967) that the consumption of fats is related to the mortality from colon cancer is supported by the results of a study of Japanese (Wynder *et al.*, 1969). This study showed that the increase in mortality from colon cancer observed in Japanese is associated with the recent Westernisation of the diet which includes a higher caloric intake in the form of fats. This finding is supported by the known increase in colon cancer among Japanese in USA who adopted the American diet (Haenszel and Kurihara, 1968). In examining more recent data, Armstrong and Doll (1975) also reported positive correlations between dietary fat consumption and the incidence and mortality from cancers of the breast, uterus, ovary, prostate, testis, colon and rectum.

The cancer mortality data of Segi *et al.* (1969) has recently been compared by Carroll and Khor (1975) with data published by the Food and Agricultural Organization of the United Nations (1971) on the consumption of dietary fats in different countries. A highly significant correlation was found between mortality from breast cancer and the consumption of fats ($r = 0.935$) (Figure 1). Only two countries, South Africa and Israel, deviated appreciably from the general trend. The results for these countries are misleading as the cancer mortality for South Africa is based on the European population only and that for Israel is based only on the Jews,

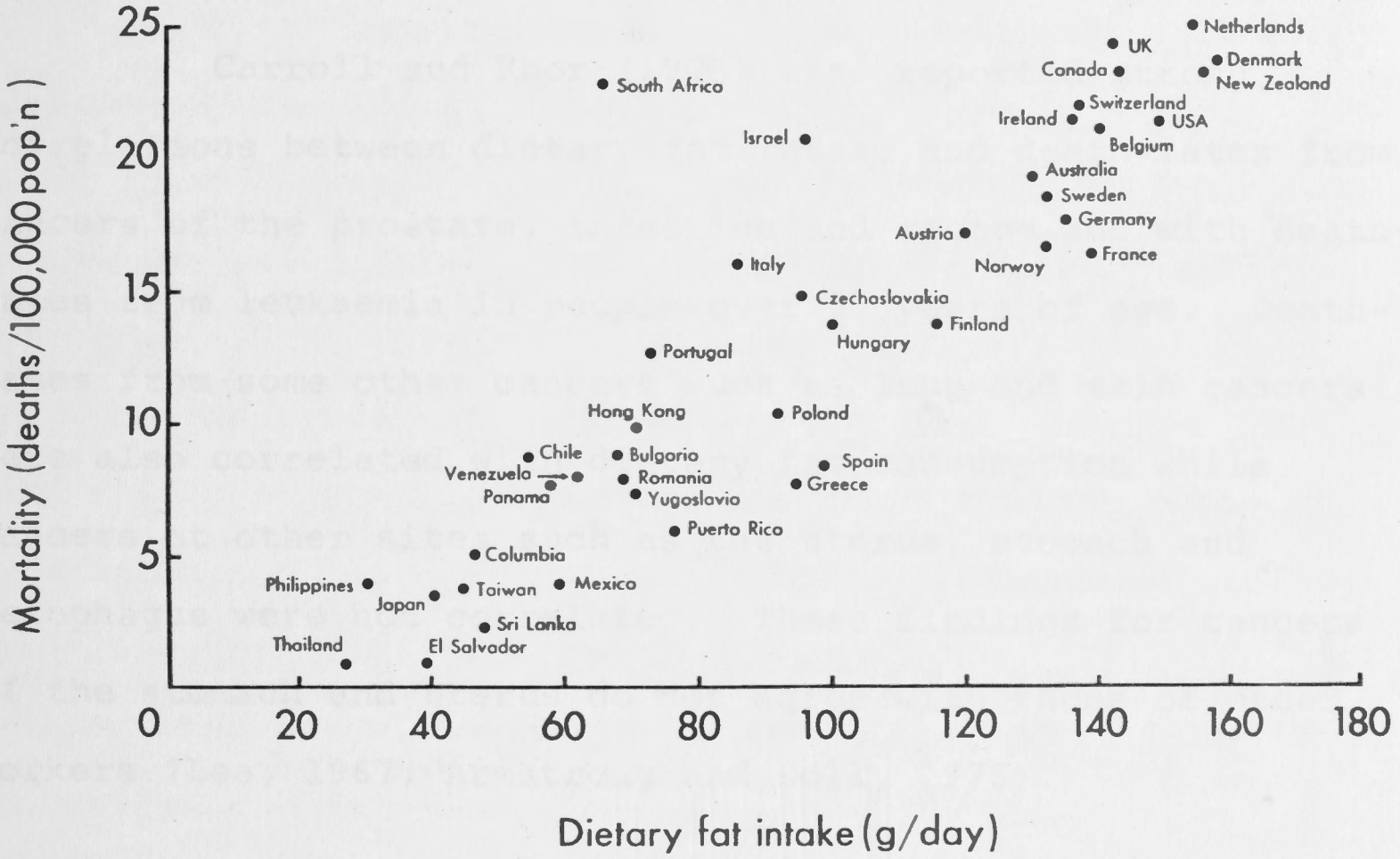


FIGURE 1. *Correlation between per capita consumption of dietary fat and age-adjusted mortality from breast cancer in women.* From Carroll and Khor, 1975.

whereas the fat consumption figures are calculated for each country as a whole. The age-adjusted incidence of breast cancer in the white population of South Africa is much greater than in the non-white, and Jews in Israel show a much higher incidence than non-Jews (Doll *et al.*, 1970).

Carroll and Khor (1975) also reported strong correlations between dietary fat intake and death-rates from cancers of the prostate, intestine and rectum and with death-rates from leukaemia in people over 55 years of age. Death-rates from some other cancers such as lung and skin cancers were also correlated with dietary fat consumption while cancers at other sites such as the uterus, stomach and oesophagus were not correlated. These findings for cancers of the stomach and uterus do not agree with those of other workers (Lea, 1967; Armstrong and Doll, 1975).

There are no satisfactory data on the consumption of various types of fat in different countries or by defined groups of people, so it is not possible to make comparisons between the consumption of different fats and the incidence of various cancers. However, epidemiological data on humans does indicate that mortality from breast cancer is more strongly correlated with total dietary fat consumption than with animal fat, but that the intake of plant fat is not correlated with mortality from breast cancer (Carroll, 1975). Although plant fat may be expected to be more un-saturated than animal fat, some plant fats such as coconut

oil are relatively saturated and others may be hydrogenated for edible use. Therefore, the data on the consumption of plant fat does not provide a reliable guide to possible differences between effects of saturated and unsaturated fats on human cancers.

The analysis by Pearce and Dayton (1971) of the incidence of cancers in groups of people on an 8 year controlled clinical trial designed to examine the efficacy of polyunsaturated fats in reducing coronary heart disease, highlighted the possibility that polyunsaturated fats in the diet may increase the incidence of cancer. In this trial the difference in the incidence of neoplasms between those on the saturated fat diet and those on the polyunsaturated fat diet was not statistically significant ($P = 0.06$). The study was carried out with 846 men in an older age group and the increased number of carcinomas was not localised to any particular site. No such differences have been found in other diet-coronary heart disease trials (West and Redgrave, 1974). However, the lack of differences in these trials does not necessarily mean that there is no link between dietary poly-unsaturated fat and cancer or that further investigation is not warranted. The trials were not designed to examine the hypothesis that dietary fat may increase the incidence of death from causes other than coronary heart disease. It is interesting to note that even if there has been a reduction in deaths from coronary heart disease, this has not been accompanied by lower overall rates of mortality in the diet-

coronary heart disease trials so far carried out. Some of these trials have been criticised because of shortcomings and inconclusive results which make it difficult to draw meaningful conclusions from them (Halperin *et al.*, 1973). It would also be difficult to carry out a worthwhile trial to examine the role of dietary fats in carcinogenesis, especially as the fat almost certainly does not act as a carcinogen but as a cocarcinogenic agent. It is quite possible that substances associated with dietary fats could be involved in carcinogenesis, but the provision of satisfactory standards should enable these risks to be controlled (West and Redgrave, 1974).

The interpretation of statistical correlations such as those discussed here should be approached with caution as they do not reveal cause and effect relationships and are, by themselves, inconclusive. There may also be difficulties in collecting reliable epidemiological data and the association between variables may be coincidental. However, when suggestions from epidemiological studies are supported by evidence from experiments with animals, more serious consideration is required. Because there is the suggestion from data on humans that various types of dietary fats may promote carcinogenesis, it is worthwhile examining evidence from experimental work with animals.

2. Carcinogenesis in animals

The effect of dietary fat on carcinogenesis in

experimental animals has recently been extensively discussed by Carroll and Khor (1975). There is general agreement from experiments carried out over the last 30 years that rats and mice maintained on high fat diets are more prone to develop mammary and skin tumours than animals fed low fat diets. Spontaneous as well as induced tumours appear to be more prevalent when animals are fed high fat diets. Tannenbaum (1945) concluded that these effects were due to a specific action of the dietary fat rather than to a calorific effect. This conclusion was based on the observation that the incidence of mammary and skin tumours was altered by changes in the fat content of the diet whereas caloric restriction *per se* inhibited other tumours as well (Tannenbaum, 1942). Furthermore, this inhibitory effect could be reversed by increasing the fat content of the diet while maintaining the restricted caloric intake (Tannenbaum, 1945). It can be suggested that the effect of changing the fat content of the diet is due to the concomitant changes in other dietary constituents. However, changing the proportions of carbohydrate and casein while keeping the fat content constant has been shown to have no effect on the incidence of spontaneous mammary tumours or 3,4-benzpyrene-induced sarcomas and skin tumours in mice (Tannenbaum and Silverstone, 1949).

The work of Carroll's group (Gammal *et al.*, 1967) has suggested that the type of fat may also influence carcinogenesis. The incidence of mammary tumours induced by 7,12-dimethylbenz(*a*)anthracene (DMBA) was increased by feeding rats

a high fat diet containing polyunsaturated fat rather than a low fat diet or a high fat diet containing saturated fat. Carroll and Khor (1971) have assessed the effect of a number of dietary fats on the incidence of mammary tumours in DMBA-dosed rats. There tended to be more tumours per rat when rats were fed unsaturated fats and this was reflected in a trend towards higher tumour yields with increased unsaturation of the diets. Of the total number of tumours, 95% were adenocarcinomas and the higher yields on diets containing unsaturated fats were confined to this type of tumour. Rape-seed oil was an exception to this association between unsaturation and tumour yield. This may have been due to the high content of the mono-unsaturated C_{20} and C_{22} fatty acids in this oil. Rape-seed oil also had a lower digestibility than the other fats and oils, and is known to depress growth in rats (Thomasson and Boldingh, 1955).

Some workers (Nestel, 1974) have dismissed the suggestion that polyunsaturated fats may increase the incidence of tumours in animals by quoting the work of Schramm. Schramm (1961a) has studied the effect of feeding ethyl esters of various fatty acids on the incidence of 3-methyl-4-dimethylaminoazobenzene-induced liver tumours in rats. While the feeding of ethyl stearate and ethyl linoleate did not influence the incidence of tumours and ethyl oleate slightly reduced the incidence, ethyl linolenate prevented the formation of tumours even after 18

months administration of the carcinogen. Ethyl linolenate feeding also reduced the incidence of tumours of the external auditory meatus in rats fed 4-dimethylaminostilbene (Schramm, 1961b), but did not reduce the incidence of tumours induced in rats by 3-methylcholanthrene and in mice by either DMBA or urethane (Schramm, 1961c). There is evidence to suggest that the effects of fatty acid ethyl esters reported by Schramm are due not to the fatty acid moieties but to the ethyl group. Thus Castagna *et al.* (1972) have shown that the feeding of ethionine to rats, while not inducing hepatomas itself, did reduce the incidence and growth of hepatomas induced by 4-dimethylaminoazobenzene and reduced the binding of its metabolites to DNA. The authors suggested that one possible mode of action may be an alteration in molecular receptors on DNA. This hypothesis is supported by the work of Swann *et al.* (1971) which showed that the feeding of ethionine to rats produces ethylation at the 7-position of guanine in liver DNA.

This survey will now examine the effect of dietary fats on the metabolism of chemical carcinogens followed by a discussion of the two stage theory of tumour induction as it relates to the effect of dietary fats. The changes induced by dietary fats in the structure and function of biological systems which may be involved in carcinogenesis will then be examined. These include changes in membrane structure and function, immunocompetence, DNA repair potential and endocrine function.

3. Metabolism of chemical carcinogens

As suggested by Dao *et al.* (1959) it is possible that mammary adipose tissue functions as a storage depot for carcinogenic polycyclic hydrocarbons and that its proximity to the glandular epithelium allows prolonged exposure to the carcinogen. Tannenbaum and Silverstone (1957) postulated that dietary fat may produce changes in the lipid composition of tissues such as the mammary gland and that such changes may affect the rate of transport of carcinogens to their site of action. To test this hypothesis Gammal *et al.* (1968) examined the effect of various diets on the concentration of DMBA in the mammary tissue of DMBA-dosed rats. They found that there was a rapid rise in the concentration of DMBA which reached a peak 6 hours after its administration to animals fed diets containing 0.5 or 20% (w/w) corn oil. However, in animals fed a 20% (w/w) coconut oil diet, the peak concentration was delayed to 12 hours and was of a smaller magnitude than that observed in the two corn oil groups. It is difficult to interpret such observations as the optimum concentration of DMBA required to elicit a carcinogenic response is not known. The observation that tissue concentrations of DMBA were the same in animals fed either the 0.5 or 20% corn oil diet, whereas the tumour yield was enhanced in the latter (Gammal *et al.*, 1967), suggests that additional factors are involved in the process.

It is possible that dietary fats could influence the

microsomal metabolism of chemical carcinogens. It is thought that as many as 90% of cancers seen in man are caused by environmental factors, mostly chemicals (Maugh, 1974). Many chemical carcinogens must be activated by metabolic alteration before exerting a carcinogenic action. In most cases the enzymes involved in this activation are the microsomal mixed function oxygenases which convert many precarcinogens to their active electrophilic derivatives (Miller, 1970; Farber, 1973). In addition to producing more active derivatives, microsomal enzymes may also produce less active derivatives (Farber, 1973). The balance between the activating and deactivating reactions is important in determining the carcinogenic effects of many compounds. This might best be illustrated by considering the metabolism of 2-acetylaminofluorene (AAF) which can occur by at least two pathways (Miller and Miller, 1971). N-hydroxylation of AAF and subsequent N-sulphation results in the formation of what has been termed the ultimate carcinogen, while ring hydroxylation of AAF and N-hydroxy AAF appears to be a process of deactivation. Thus, much of the carcinogenic action may be lost in deactivation reactions, but once enough of the ultimate carcinogen has been formed and has reacted with cellular macromolecules for a sufficient time, the carcinogenic process will be initiated.

In vitro studies have shown that the cytotoxicity and rate of malignant transformation caused by a number of

hydrocarbons can be enhanced by induction of mixed function oxygenase enzymes (Gelboin, 1972; Marquardt and Heidelberger, 1972). It should not be assumed that the biological effects of altered mixed function oxygenase activity in tissue culture will predict the outcome of similar changes in the intact animal. The mixed function oxygenase enzymes catalyse the formation of more polar compounds which can be further metabolised and excreted. In cell culture, metabolites remain in the culture medium and may cause changes that would not have occurred if they had been transported and excreted by the animal. In this case, the results of *in vivo* studies do differ from those which might be predicted from *in vitro* studies.

A considerable number of experiments have shown that increased activity of the mixed function oxygenase enzymes is accompanied by an inhibition of carcinogenesis induced in various tissues by a number of chemical carcinogens (Wattenberg, 1975). This may result from the conversion to a less carcinogenic derivative, the increased transport and excretion of the carcinogen, the loss of some of the ultimate carcinogen due to saturation of binding sites on macromolecules or from secondary reactions. Wheatley (1968) has suggested that the observed enhancement of DMBA-induced carcinogenesis in the mammary gland following treatment with an inhibitor of the microsomal enzyme system is due to an increase in the effective dose of the carcinogen in the target tissue. Gelboin *et al.* (1970)

have reported that 7,8-benzoflavone, an inhibitor of aryl hydrocarbon hydroxylase, has an inhibitory effect on DMBA-induced tumorigenesis in mouse skin. The reason for this discrepancy with the bulk of work on this topic is not obvious, but the results of Gelboin and his colleagues may be specific to the compounds and tissue system employed.

The activity of the mixed function oxygenase system has been shown to be altered not only by various drugs, such as some barbiturates, but also by dietary modifications such as changes in the lipid composition of the diet. Liver microsomes from rats fed diets containing increased amounts of corn oil contain greater concentrations of cytochrome P-450 (Marshall and McLean, 1971; Norred *et al.*, 1971; Norred and Wade, 1972) which is a constituent of the mixed function oxygenase system (Jansson and Schenkman, 1975). They also metabolise at a faster rate a variety of drugs including hexobarbital, heptachlor, aniline and ethyl morphine (Caster *et al.*, 1970; Norred *et al.*, 1971; Norred and Wade, 1972). The phenobarbital-induced metabolism of hexobarbital and aminopyrine and the concentration of cytochrome P-450 are also increased when rats are fed diets containing polyunsaturated fats such as corn, linseed and herring oils rather than diets containing more saturated fats such as beef fat or coconut oil (Marshall and McLean, 1971; Century, 1973). It is possible that either the degree of unsaturation of the dietary lipids or the propensity to form lipid peroxides may have been responsible for the

results of these experiments.

4. The two stage theory of tumour induction

The results of experiments mainly with skin tumours have given rise to the concept that tumour production is a process involving two discontinuous stages (Friedewald and Rous, 1944; Berenblum and Shubik, 1947). The first, or initiation, stage involves the carcinogenic event in which normal cells are transformed into neoplastic cells by interaction with a carcinogenic agent. This process is thought to be rapid and irreversible although, as discussed later, DNA repair can occur from an intermediate stage. Only one exposure to a carcinogen is deemed necessary and initiated cells can be converted to tumours long after the exposure to the carcinogen (Salaman and Roe, 1964). The second, or promotional, stage involves the process whereby initiated cells multiply to finally form an established tumour (Berenblum, 1954). It has been suggested that it is during this stage that many of the known effects of environmental factors which influence carcinogenesis exert their effect (Rusch *et al.*, 1945). This stage may involve a progressive selection of cell populations for characteristics such as autonomous growth, malignancy and resistance to cytotoxicity (Farber, 1970, 1973). An established tumour may also progress to more malignant forms by irreversible changes in the characters of its cells (Foulds, 1954, 1969). The initial causative stimulus in carcinogenesis is referred to

as the initiating action and any subsequent stimulus which leads to, or enhances, tumour development is referred to as a promoting action (Friedewald and Rous, 1944).

Lavik and Baumann (1941) and Tannenbaum (1942, 1944) considered that dietary fat may exert a promoting action on carcinogenesis. They referred to the cocarcinogenic action of dietary fat being more effective in increasing the tumour incidence when high fat diets were fed after application of the carcinogen than during carcinogen application. In order to interpret the effect of various dietary fats on the incidence of DMBA-induced tumours in terms of the two stage theory, Carroll and Khor (1970) examined the effect of diets containing either 0.5 or 20% corn oil, fed to rats before and after the administration of DMBA. Rats fed the high fat diet after DMBA administration had a higher tumour incidence than those fed the low fat diet, regardless of the type of fat fed before the DMBA was administered. Carroll and Khor (1975) have also shown that the yield of mammary tumours in DMBA-dosed rats was enhanced if the rats were transferred to a high corn oil diet 1 or 2 weeks after giving the DMBA, but if the transfer was delayed for 4 weeks little or no enhancement was seen. Thus the evidence suggests that the diet fed in the first month after dosing with the carcinogen is critical in influencing the tumour incidence. As discussed below, changes during this period in the structure and function of membranes and in immunocompetence may be important.

5. Membrane structure and function

It has been shown that the fatty acid composition of various tissues of monogastric animals reflects the fatty acid composition of dietary lipids (Tove and Smith, 1960; Ostwald *et al.*, 1962; Erwin and Sterner, 1963; Leat, 1963; Beare and Kates, 1964; Stokes and Walker, 1970; Bouchard and Brisson, 1971). Similar observations have been made in ruminants provided that the dietary fats are protected against hydrogenation by ruminal micro-organisms (Ogilvie *et al.*, 1961; Erwin *et al.*, 1963; Cook *et al.*, 1972). The fatty acid compositions of a number of subcellular organelles have been determined, but there have been differences between the results reported by various workers (Macfarlane *et al.*, 1960; Marco *et al.*, 1960; Schwarz *et al.*, 1961; Getz *et al.*, 1962; Veerkamp *et al.*, 1962). As suggested by van Deenen (1965) such differences may have been produced by different dietary conditions. This view is supported by the work of Witting *et al.* (1961), which showed that the fatty acid composition of rat liver mitochondrial lipids is readily altered by varying the fatty acid composition of the diet. Similarly, the fatty acid composition of rat erythrocyte ghosts reflects the unsaturated fatty acid composition of the dietary lipids (Witting *et al.*, 1961; Walker and Kummerow, 1963, 1964; Bloj *et al.*, 1973). Although studies of diet-induced changes in the fatty acid composition of such subcellular organelles have been reported, similar studies on plasma membranes are not evident.

Changes in the fatty acid composition of plasma membranes are of physiological significance. Artificial and natural membranes containing unsaturated lipids have been shown to be more permeable than those containing more saturated lipids. Liposomes formed from highly unsaturated lipids, rather than saturated lipids, are more permeable to neutral amino acids (Klein *et al.*, 1971), non-electrolytes such as glycerol, glucose and erythritol (de Gier *et al.*, 1968; Demel *et al.*, 1968; McElhaney *et al.*, 1970; Demel *et al.*, 1972), and ions such as Rb^+ and K^+ (de Gier *et al.*, 1970). Similar results have been found for non-electrolytes and ions with intact cells of the bacterium, *Acholeplasma laidlawii* in which the composition of the membrane lipids can be altered by varying the fatty acid composition of the growth medium (McElhaney *et al.*, 1970; de Kruyff *et al.*, 1973a; van der Neut-Kok *et al.*, 1974).

As blood levels of cell nutrients are relatively constant throughout an animal, it has been suggested by Holley (1972) that cells of different tissues could possibly control their growth by selectively altering the availability of nutrients to the interior of the cell. Similarly, the survival and subsequent growth and division of neoplastic cells could be favoured by this change in membrane composition and permeability. It is interesting that the fatty acid composition of lipids from hepatomas and mammary tumours differ from those of the normal tissues (Araki *et al.*, 1974;

Tan *et al.*, 1975) and that the plasma membranes of hepatoma cells have an altered fatty acid composition (van Hoeven and Emmelot, 1973; Dnistrian *et al.*, 1976). However, the reported differences in fatty acid composition have not been consistent.

Permeability is not the only characteristic of membranes altered by changes in fatty acid composition. The changes in the membrane lipid phase transition, responsible for the changes in permeability (de Kruyff *et al.*, 1973a), also produce breaks in the Arrhenius plots of the activities of lipid-associated membrane-bound enzymes (de Kruyff *et al.*, 1973b). It is possible that such differences in enzyme activity could favour the development of neoplastic cells.

6. The immune system

It is now widely accepted that immune reactions can be generated in response to tumour-associated antigens presented by at least some spontaneous tumours in man and experimental animals (Klein and Oettgen, 1969). Immunological destruction of tumour cells *in vivo* is primarily the function of sensitised lymphoid cells, although specific circulating antibody may play a minor role (Bansal and Sjögren, 1974). Assuming such a defence mechanism to be of physiological consequence to the potential tumour-bearing host (Figure 2), an influence of dietary fats on immunological processes could lead to an involvement of such fats in carcinogenesis.

Much of the work on the role of various dietary fats in immunological processes has originated from studies of multiple sclerosis. It has been suggested that the development of this disease depends in some way on the recognition by sensitised cells of the putative encephalitogenic antigen. Using an experimental model for multiple sclerosis, Clausen and Møller (1967) found that rats bred and raised on a diet deficient in polyunsaturated fatty acids were more susceptible to allergic encephalomyelitis than rats bred and raised on a normal diet. Millar *et al.* (1973) carried out a double-blind study with patients suffering from multiple sclerosis and found a slight improvement in the clinical course of the disease over a 2 year period when patients were fed a diet rich in linoleic acid rather than oleic acid. Further support for an immuno-inhibitory action of polyunsaturated fatty acids comes from studies of graft survival. It has been shown that either feeding or injection of linoleic acid prolongs skin allograft survival in rodents (Mertin, 1974; Ring *et al.*, 1974; Hughes *et al.*, 1975; Mertin, 1976). Ingestion of sunflower-seed oil or an oil rich in linoleic and γ-linolenic acids has also been claimed to be beneficial as an adjunct to immunosuppressive therapy following human renal transplantation (Uldall *et al.*, 1974, 1975).

Studies using *in vitro* techniques have also indicated that polyunsaturated fatty acids are immuno-inhibitory. Mertin and his colleagues (Mertin, 1973;

Mertin *et al.*, 1973, 1974), using the macrophage electrophoretic mobility test, have shown that either linoleic or arachidonic acid, but not oleic acid, suppress the antigen-induced lymphocyte response. As the addition of the fatty acid after the antigen had no effect, they concluded that the polyunsaturated fatty acids interfered with the recognition by lymphocytes of antigens such as PPD (purified protein derivative of tuberculin), encephalitogenic factor and thyroid antigen. The lymphocyte transformation test has also been used to substantiate the results obtained using the macrophage electrophoretic mobility test. Using this test, Mertin *et al.* (1974) were able to demonstrate the same order of effectiveness of the fatty acids (oleic < linoleic < arachidonic) in inhibiting the PHA (phytohaemagglutinin) and PPD-induced lymphocyte responses. In a later publication, Mertin and Hughes (1975) showed that the saturated fatty acids, palmitic and stearic acids, could also inhibit the *in vitro* antigen-induced lymphocyte response. In titration experiments, the inhibition by palmitic acid showed only random variation while the inhibition by stearic acid reached a plateau when the fatty acid was added to the culture medium at a calculated final concentration of 0.08 mg/ml. The rates of increase in inhibition by the unsaturated fatty acids did not decrease over the concentration range (0.02 - 0.16 mg/ml) tested. However, these calculated concentrations probably bear little resemblance to the actual concentrations of fatty

acids in the culture media as the fatty acids added were not bound to albumin. This is in fact indicated by the authors who found that the measured concentration of linoleic acid in the culture medium was approximately half that added. Mertin and Hughes (1975) also examined the specificity of the fatty acid-induced inhibition by comparing the inhibitory effect of each fatty acid on antigen-stimulated and non-stimulated lymphocytes. Only the polyunsaturated fatty acids, linoleic and arachidonic acids, exhibited a clear difference in inhibition of stimulated and non-stimulated cells, suggesting that these fatty acids exert a specific, inhibitory action restricted to activated lymphocytes.

Offner and Clausen (1974) studied the effect of fatty acids and prostaglandins on PHA and PPD-induced lymphocyte transformation by measuring the incorporation of myo-(2-^{3}H)-inositol into phosphatidylinositol. Linoleic and arachidonic acids and prostaglandins E_1 and E_2 (PGE_1 and PGE_2, respectively) inhibited the lymphocyte response to a much greater extent than did oleic acid. Thus the inhibitory effects of the polyunsaturated fatty acids in these *in vitro* immunological test systems may be mediated through the prostaglandins which are synthesised by membrane-bound prostaglandin synthetase from polyunsaturated fatty acids (Ramwell *et al.*, 1968). This is supported by work which shows that the prostaglandin-stimulated synthesis of cyclic 3', 5'-adenosine monophosphate (de Boer *et al.*, 1973) regulates

the expression of immediate and delayed hypersensitivity (Henney *et al.*, 1972; Lichtenstein *et al.*, 1972; Strom *et al.*, 1973) and that PGE_1 suppresses homograft rejection in mice (Quagliata *et al.*, 1973).

Tan *et al.* (1974) have shown that the rate of synthesis of prostaglandins and the concentration of PGE_2 are both greater in DMBA-induced mammary tumours than in normal mammary tissue of rats. Several other types of tumours have also been shown to produce increased amounts of prostaglandins (Sykes and Maddox, 1972; Tashjian *et al.*, 1972; Humes and Strausser, 1974). As PGE_1 and PGE_2 inhibit cell-mediated immune responses it is conceivable that the increased rates of synthesis of these prostaglandins by tumours may provide tumour cells with some protection against immunological destruction. This hypothesis is supported by *in vitro* and *in vivo* studies which have shown that chemical or virus-induced tumours inhibit immune responses in mice bearing the tumours (Plescia *et al.*, 1975). In addition, indomethacin and aspirin, inhibitors of prostaglandin synthesis, blocked the immunosuppression *in vitro* and retarded tumour growth *in vivo*.

PGE_1 and PGE_2 are synthesised from linoleic acid through the intermediate dihomo-γ-linolenic acid and from arachidonic acids, respectively (Ramwell *et al.*, 1968). Increases in the dietary intake of linoleic or arachidonic acids may therefore lead to increases in the synthesis of

these prostaglandins (Thomasson, 1969). Thus, it is possible that increased concentrations of prostaglandin may have been wholly or partly responsible for the increased tumour incidence observed by Gammal *et al.*(1967) in DMBA-dosed rats fed a diet containing corn oil (62% of the fatty acid being linoleic acid) compared to rats fed a diet containing more saturated fat.

There has been considerable interest in the involvement of brown fat in immunosuppression. The experiments by Sidky *et al.* (1969) showed that brown adipose tissue from hibernating ground squirrels depresses antibody production by hamster spleen cells in tissue culture. The inhibitory activity was later found to be extracted by lipid solvents and to be associated with brown fat from other animals capable of intensive thermogenic utilisation of their brown fat (Sidky *et al.*, 1972). Later Janković and his colleagues (Janković *et al.*, 1974a, 1974b, 1974c) showed that the Arthus and delayed hypersensitivity reactions were increased in neonatally brown fat adipectomised rats and depressed in rats injected subcutaneously with lyophilised brown adipose tissue from day-old rats or 21 day old rats kept for 7 days at 12°C. There was no effect of the brown adipose tissue on antibody production and white adipose tissue was without effect in any of the systems. Thus it appears that brown adipose tissue depresses at least humoral immunity in hibernating animals and cell-mediated

immunity in non-hibernating animals. It seems difficult to accept the suggestion of Mertin *et al.* (1974) that there is a relationship between these findings with brown adipose tissue and their work on the immunosuppressive effect of polyunsaturated fatty acids, as the fatty acids of brown adipose tissue are more saturated than those of white adipose tissue (Clément and Meara, 1951; Fawcett, 1952; Spencer and Dempster, 1962). Perhaps the immuno-inhibitory activity of brown adipose tissue is due to a factor related to its highly developed sympathetic nervous system (Steiner, 1972). Janković and Isaković (1973) have suggested that noradrenalin may be involved, but this would not explain the activity of the chloroform extracts of brown adipose tissue described by Sidky *et al.* (1972) as less than 7% of the noradrenalin would be extracted by such a procedure (West and Hopkins, unpublished observations).

7. DNA repair

The DNA repair systems provide cells with a considerable degree of protection against the actions of carcinogens and mutagens (Figure 2). There is at least one disease where a reduced DNA repair potential is associated with a high incidence of cancer. Xeroderma pigmentosum is an autosomal recessive disease causing excessive sensitivity to sunlight and a high incidence of skin cancer (McKusick, 1975). It is characterised by the inability of skin fibroblasts to repair damage to DNA bases, although breaks in the

DNA chain are readily repaired. There is therefore a defect early in the repair sequence when the base damage is recognised and the polynucleotide chain broken by an endonuclease (Cleaver, 1968, 1969; Setlow *et al.*, 1969).

Bloom's syndrome (Bloom, 1966; German, 1969), is another disease causing increased sensitivity of the skin to sunlight and a high incidence of leukaemia and cancer of the gastrointestinal tract. Lymphocytes and fibroblasts from these patients show many metaphase chromosomal aberrations including a quadriradial configuration typical of the disease. Unfortunately, the enzymatic defect responsible for Bloom's syndrome remains unidentified, but it is probably concerned with chromosomal metabolism (German and La Rock, 1969; German, 1972). People suffering from Fanconi's anaemia (Fanconi, 1967) also have a high incidence of leukaemia and an abnormally high frequency of chromosomal aberrations. The Louis-Bar syndrome (Louis-Bar, 1941) is characterised by chromosomal aberrations and a more frequent occurrence of cancer, often arising in the lymphoid reticular tissues, than is found in the general population (Peterson *et al.*, 1966; Swift *et al.*, 1976). Although chromosomal aberrations are seen in these conditions, other factors, such as reduced immunocompetence, could play a role in the development of these cancers (German, 1972).

An increased incidence of chromosomal abnormalities is also seen in groups of the population exposed to

extraordinary environmental circumstances which cause a high incidence of cancer among those exposed. Briefly, chromosomal aberrations have been observed in the lymphocytes of survivors of the atomic bomb blasts in Japan (Bloom *et al.*, 1966, 1967; Ishihara and Kumatori, 1967); in the lymphocytes of X-ray irradiated arthritic patients (Buckton *et al.*, 1962); among children who during their infancy received X-ray therapy to shrink the thymus (Simpson *et al.*, 1955); among practicing radiologists (Seltser and Sartwell, 1965) and among factory workers who inhaled benzene occupationally for prolonged periods of time (Vigliani and Saita, 1964; Tough and Court Brown, 1965).

It is therefore possible that the DNA repair systems may be important in protecting cells from the effects of carcinogenic agents. Environmental factors such as diet could alter the efficacy of these systems and could therefore be important in determining the incidence of various cancers.

8. Endocrine function

Epidemiological data on humans have revealed correlations between dietary fat and the incidence of hormone-sensitive cancers such as cancer of the breast and prostate (Carroll and Khor, 1975). It is possible that any causal relation between dietary fats and cancer in these tissues may involve an alteration of the hormonal environment (Wynder *et al.*, 1971; MacMahon *et al.*, 1973; Berg, 1975).

Evidence on the effect of dietary factors on hormone metabolism is scarce, although it is known that gross undernutrition alters the metabolism of corticoids, androgens and oestrogens (Zumoff *et al.*, 1975). Obesity in women, which is associated with an increased risk of developing breast cancer, is also accompanied by alterations in oestrogen and androgen metabolism (Lipsett, 1975).

Some rat mammary tumours are also hormone dependent in that both oestrogen and prolactin appear to be necessary for continued tumour growth (McGuire *et al.*, 1974; Bradley *et al.*, 1976). Chan and Cohen (1975) have suggested that the growth of these tumours is regulated by the ratio of the concentrations of prolactin to oestrogen in the serum. High fat intake, which raises the concentration of serum prolactin in rats at proestrus-oestrus (Chan *et al.*, 1975), may thereby promote mammary tumour cell growth. Administration of the antiprolactin drug, 2-brom-α-ergocryptine to rats has also been shown to eliminate the tumour-promoting effect of a high fat diet (Chan and Cohen, 1974). Administration of the anti-oestrogen drug, nafoxidine hydrochloride, did not eliminate this differential effect of the low and high fat diets on the tumour incidence.

9. Conclusion

The term cancer embraces a heterogeneous group of diseases characterised by the ability of the affected tissues

to overcome the normal tissue growth control mechanisms and to invade the surrounding tissues. The factors determining the pathogenesis of cancer are many and their interplay is intricate. The transformation of normal cells into neoplastic cells, their survival and their subsequent proliferation to form a tumour capable of causing death of the host is a complex phenomenon. The final outcome depends on the efficacy of the various systems of initiation, promotion and defence. A schematic representation of mechanisms involved in carcinogenesis is shown in Figure 2. It is with reference to this simplified, but helpful, approach to carcinogenesis that this thesis examines the influence of the nature of dietary fat on carcinogenesis in animals. The possible modes of action by which dietary fats may influence carcinogenesis are studied by investigating the effect of dietary fats on the activities of several biological mechanisms involved in carcinogenesis.

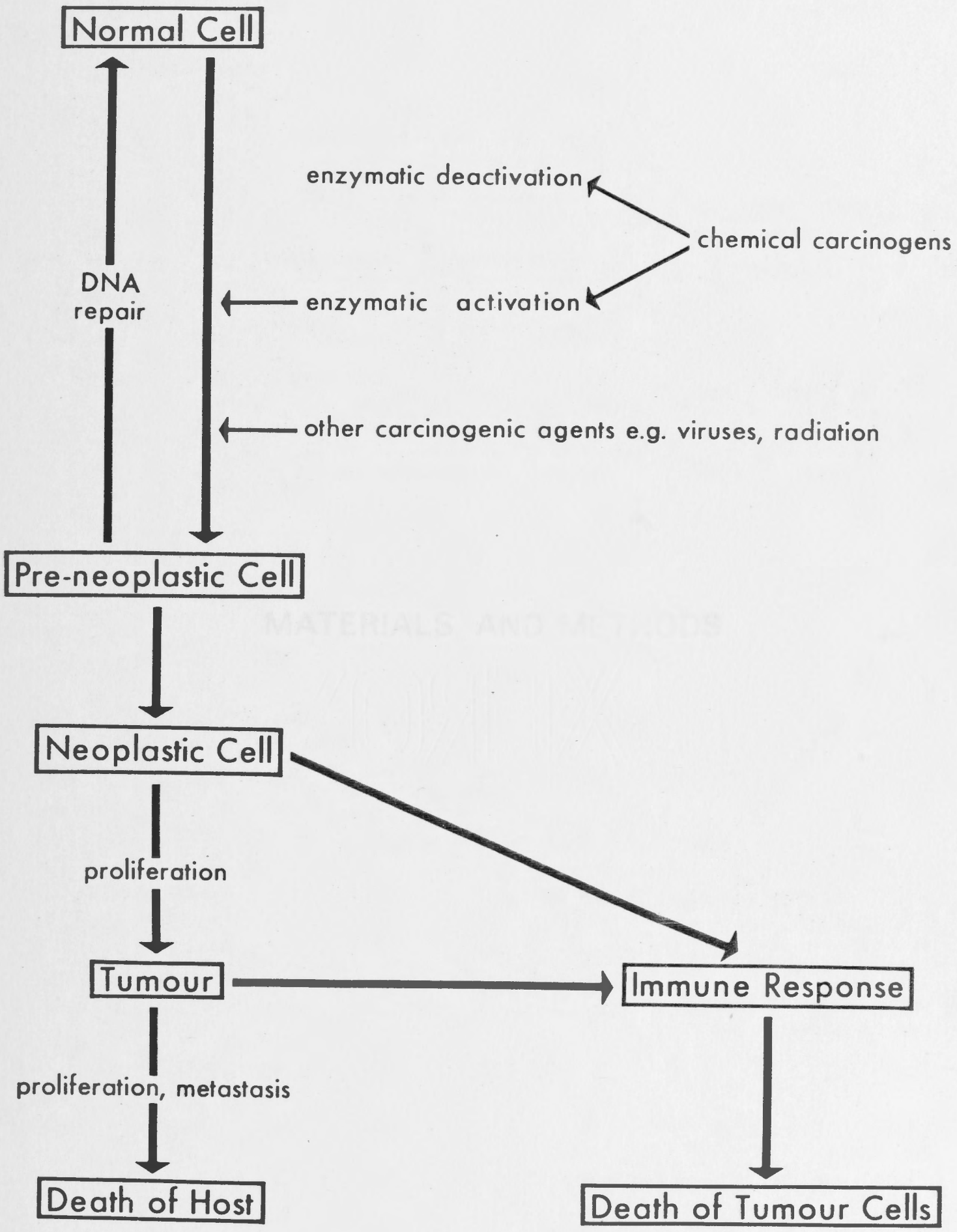


FIGURE 2. *Schematic representation of mechanisms involved in carcinogenesis.*

MATERIALS AND METHODS

1. Animals

(a) Rats Weanling female Wistar-derived rats were bred in the Animal Breeding Establishment of the Australian National University.

(b) Mice Virgin female CBA/h mice and virgin C3HA^{vy}fB mice of both sexes were bred in the Animal Breeding Establishment of the Australian National University. The C3HA^{vy}fB mice were derived from stock obtained from Dr G. Vlahakis of the National Cancer Institute, Bethesda, Md 20014, USA. The C3HA^{vy}fB strain originated from a C3HA^{vy} litter delivered by caesarean section and foster-nursed on C57BL mice to prevent infection with mammary tumour virus which is present in the milk of C3HA^{vy} mice (Heston and Vlahakis, 1968). The C3HA^{vy}fB mice have a 90 to 100% incidence of spontaneous tumours of both the liver and mammary glands (Heston and Vlahakis, 1968; Vlahakis *et al.*, 1970). The high incidence of tumours in this strain appears to be determined by the A^{vy} gene which also determines the yellow coat colour and the increased body weight (Vlahakis *et al.*, 1970). The presence of nodule-inducing virus (Nandi, 1966) and the hormonal stimulation of pregnancy are other factors which may interact to produce the high incidence of mammary tumours in this strain (Vlahakis *et al.*, 1970). Sabine *et al.* (1973) reported that the high incidence of tumours in C3HA^{vy} and C3HA^{vy}fB mice in USA was dramatically reduced when the mice were bred and reared in Australia. Female C3HA^{vy} mice reared in Australia, but supplied with feed and cage bedding (containing shavings from Eastern Red Cedar,

Juniperus virginiana) from USA had a similar incidence of mammary tumours as mice kept in USA. These workers showed that the cage bedding was responsible for the altered tumour incidence. Heston (1975) has recently suggested that the low incidence of tumours in the mice bred and reared in Australia was due to failure to control infestation with ectoparasites. The addition of cedar shavings to the cage bedding of animals is regularly used in USA to prevent infestation with ectoparasites. Some of the mice used in the experiments described in this thesis were examined under a dissecting microscope and were found to be free of ectoparasites. Regardless of the mode of action of cedar bedding in increasing the incidence of tumours, C3HA^{vy}fB mice are obviously highly susceptible to the action of carcinogenic and cocarcinogenic agents and are therefore well suited for use in studies of the effect of environmental factors on carcinogenesis.

2. Housing of animals

Rats and mice were housed 4 to 6 per cage and allowed free access to food and water. At the beginning of each experiment animals were arranged in treatment groups with similar mean body weights and ages. Animals which received a carcinogen or a placebo were kept in quarantine in a room with decreased air pressure during administration of the carcinogen and for 4 weeks after administration. The cages containing mice which received an intragastric dose of a carcinogen or a placebo were fitted with filter-caps to minimise contamination of the air with dust. In all experiments involving carcinogens research workers wore protective

clothing, goggles and face masks covering mouth and nose.

3. Diets

Rats and mice were maintained on a low fat pellet diet routinely used in this School or high fat diets containing sunflower-seed oil (polyunsaturated fat diet) or tallow (saturated fat diet). The low fat diet was formulated and compounded by Bunge Australia Pty Ltd, Murrumburrah, NSW 2595. The high fat diets were formulated by Rural Chemical Industries Pty Ltd, Glenorie, NSW 2157. Only the first batch of the high fat diets was compounded by Rural Chemical Industries. Later batches were compounded using facilities provided by the CSIRO Division of Plant Industry, Acton, ACT 2601. The high fat diets were stored at -15°C before use. The low fat diet was fed as pellets 1.3 cm in diameter and 1.5 to 3.5 cm long and the high fat diets as pellets 0.8 cm in diameter and 0.5 to 1.0 cm long. In later experiments the high fat diets were fed as pellets 1.0 cm in diameter and 1.5 to 2.5 cm long.

The proportions of ingredients, proximate analyses and fatty acid compositions of the diets are given in Tables 1 to 3 respectively. The low fat diet contained 4.5% fat while the high fat diets contained 18.6% fat. The major fatty acid in the polyunsaturated fat diet was linoleic acid (59% of fatty acids) while in the saturated fat diet palmitic and oleic acids accounted for about 65% of the fatty acids.

TABLE 1. Compositions of the low fat diet and high fat diets containing either polyunsaturated or saturated fat.

Ingredient	Low fat diet	High fat diets[a]	
		Polyunsaturated	Saturated
Wheat	17.51	41.4	41.4
Wheat bran	10.0	-	-
Wheat pollard	9.18	-	-
Barley	14.99	-	-
Oats	10.0	-	-
Maize	10.0	-	-
Dehydrated lucerne meal	-	5.5	5.5
Soyabean meal	4.17	32.0	32.0
Meat meal	13.9	-	-
Fish meal (65% protein)	10.0	-	-
Dicalcium phosphate	-	0.5	0.5
Lime	-	2.5	2.5
Vitamin and mineral premixes	0.25[b]	0.5[c]	0.5[c]
Methionine	-	0.1	0.1
Tallow	-	-	17.5
Sunflower-seed oil	-	17.5	-

Each value is percentage composition by weight.

[a]The sunflower-seed oil contained lauryl gallate (0.01% w/w) and butylated hydroxyanisole (0.02% w/w) as anti-oxidants. Ethoxyquin was added to the saturated fat diet at a final concentration of 0.01% w/w. The use of these anti-oxidants, which are commonly used in foods for human and animal consumption, is justified as fats and oils available commercially contain different types and amounts of added and natural anti-oxidants.

TABLE 1. (Continued):

[b]The vitamin and mineral premix supplemented the diet to the following amounts per kg total diet: vitamin A, 7333 IU; vitamin D_3, 1465 IU; vitamin E, 22 IU; vitamin B_1, 1.43 mg; vitamin B_2, 2.86 mg; vitamin B_6, 1.43 mg; vitamin B_{12}, 7.3 µg; vitamin C, 25 mg; nicotinic acid, 22 mg; pantothenic acid, 7.3 mg; iron, 50 mg; copper, 12.5 mg; cobalt, 1 mg; iodine, 1.5 mg; manganese, 75 mg; zinc, 55 mg; magnesium, 150 mg.

[c]The vitamin and mineral premix supplemented the diets to the following amounts per kg total diet: vitamin A, 13200 IU; vitamin D_3, 1320 IU; vitamin E, 53 IU; vitamin K_3, 4.9 mg; vitamin B_1, 12 mg; vitamin B_2, 27 mg; vitamin B_6, 12 mg; vitamin B_{12}, 12 µg; vitamin C, 233 mg; biotin, 66 µg; folic acid 7.3 mg; nicotinic acid, 146 mg; pantothenic acid, 43 mg; *p*-aminobenzoic acid, 49 mg; inositol, 244 mg; choline, 480 mg; iron, 70 mg, copper, 20 mg; cobalt, 50 µg; iodine, 1.6 mg; manganese, 60 mg; zinc, 60 mg.

TABLE 2. Proximate analyses of the low fat diet and high fat diets containing either polyunsaturated or saturated fat.

Component	Low fat diet	High fat diets
Protein	22.4	20.5
Fat	4.5	18.6
Fibre	5.1	4.1
Calcium	2.15	1.16
Phosphorus (available)	1.17	0.52
Phosphorus (total)	1.45	0.76
Methionine	0.43	0.40
Methionine and cysteine	0.84	0.80
Lysine (available)	1.11	1.11
Lysine (total)	1.31	-[a]
Leucine	1.38	-
Isoleucine	0.8	-
Tryptophan	0.26	-
Choline	-	0.18

Each value is percentage composition by weight.

[a]Not estimated.

TABLE 3. Fatty acid compositions of the low fat diet and high fat diets containing either polyunsaturated or saturated fat.

Fatty acids	Low fat diet	Polyunsaturated fat diet	Saturated fat diet[a]
14:0[b]	1.3±0.07	Trace[c]	3.2±0.05
16:0	20.1±0.26	9.1±0.03	28.0±0.05
16:1	-	Trace	2.3±0.05
18:0	7.4±0.08	3.5±0.10	19.3±0.05
18:1	31.0±0.31	27.2±0.20	37.1±0.15
18:2	36.0±0.26	59.0±0.05	10.1±0.05
18:3	4.2±0.17	1.2±0.05	-

Each value is the mean±SEM of 3 determinations on each of 2 samples of feed.

Units are mole % of total fatty acids.

[a] It was not possible to distinguish between the *cis* and *trans* isomers of the unsaturated fatty acids which are known to be present in tallow (Shorland, 1955).

[b] Number C atoms : number double bonds.

[c] <0.5%.

4. Chemicals

7,12-dimethylbenz(*a*)anthracene (DMBA) was obtained from Fluka, Buchs SG, Switzerland and sesame oil was obtained from Belleview Health Supplies, Chatswood, NSW 2067. Sodium pentobarbitone (Nembutal) was obtained from Abbot Laboratories Pty Ltd, Kurnell, NSW 2231.

The ethylene glycol succinate methyl silicone polymer (EGSS-X), dimethylpolysiloxane (JXR), 80/100 Gas-Chrom Q and 100/120 Gas-Chrom Q gas chromatography column packing materials were obtained from Applied Science Laboratories Inc, State College, Pa 16801, USA. The 10% diethylene glycol succinate (DEGS-PS) on 80/100 Supelcoport was obtained from Supelco Inc, Bellefonte, Pa 16823, USA. Fatty acid methyl ester standards were obtained from Nu-Chek-Prep Inc, Elysian, Minn 56028, USA.

Chemicals used as substrates in the assay of enzyme activities were obtained from the following suppliers: sodium adenosine monophosphate (sodium AMP) and glucose-6-phosphate disodium salt from Sigma Chemical Co, St Louis, Mo 63178, USA, *p*-nitrophenolphosphate (4-nitrophenyl disodium orthophosphate) and 2,6-dichlorophenolindophenol from British Drug Houses (Aust) Pty Ltd, Collingwood, Vic 3066. Analytical grade sucrose was obtained from May and Baker Pty Ltd, West Footscray, Vic 3012.

Chlorhexidine Concentrate (Hibitane) was obtained from ICI Australia Ltd, Villawood, NSW 2163. Foetal Calf Serum and Cell Culture Medium 199 were obtained from Commonwealth Serum Laboratories, Parkville, Vic 3052. Hank's Balanced Salt Solution (HBSS) and Puck's Saline A were prepared by the Department of Microbiology in this School. All other chemicals and organic solvents were of analytical grade. Organic solvents were redistilled before use.

5. Chemical methods

(a) Inorganic phosphate

Inorganic phosphate released in the assays of 5'-nucleotidase and glucose-6-phosphatase was estimated as described by Eibl and Lands (1969), using a spectrophotometer (Model 101, Hitachi Ltd, Tokyo, Japan). This spectrophotometer, if not otherwise stated, was used in all assays employing spectrophotometry. As sucrose was found to interfere with the assay, results were corrected using the relationship described in Figure 3.

(b) Cytochrome P-450

The concentration of cytochrome P-450 was determined spectrophotometrically as described by Omura and Sato (1964). An aliquot of microsomal suspension was diluted 1 in 2 with 0.1M phosphate buffer (pH 7.0). Solid sodium dithionite (1 to 2 mg) was added and the microsomal suspension divided and placed in two cuvettes of 1 cm light path. Carbon monoxide was bubbled through the sample cuvette for 20 s. The difference spectrum between the sample and reference cells was recorded from 550 nm to 400 nm using a recording spectrophotometer (Model SP8000, Pye Unicam Ltd, Cambridge CBI 2PX, England). The molar extinction coefficient for cytochrome P-450 was taken as 91 $mM^{-1}cm^{-1}$ and the results expressed as μmoles/g microsomal protein as described by Omura and Sato (1964).

(c) Protein

The concentrations of protein in zonal centrifugation fractions obtained during the preparation of hepatocyte plasma membranes were estimated from the absorbances at 280 and 260 nm.

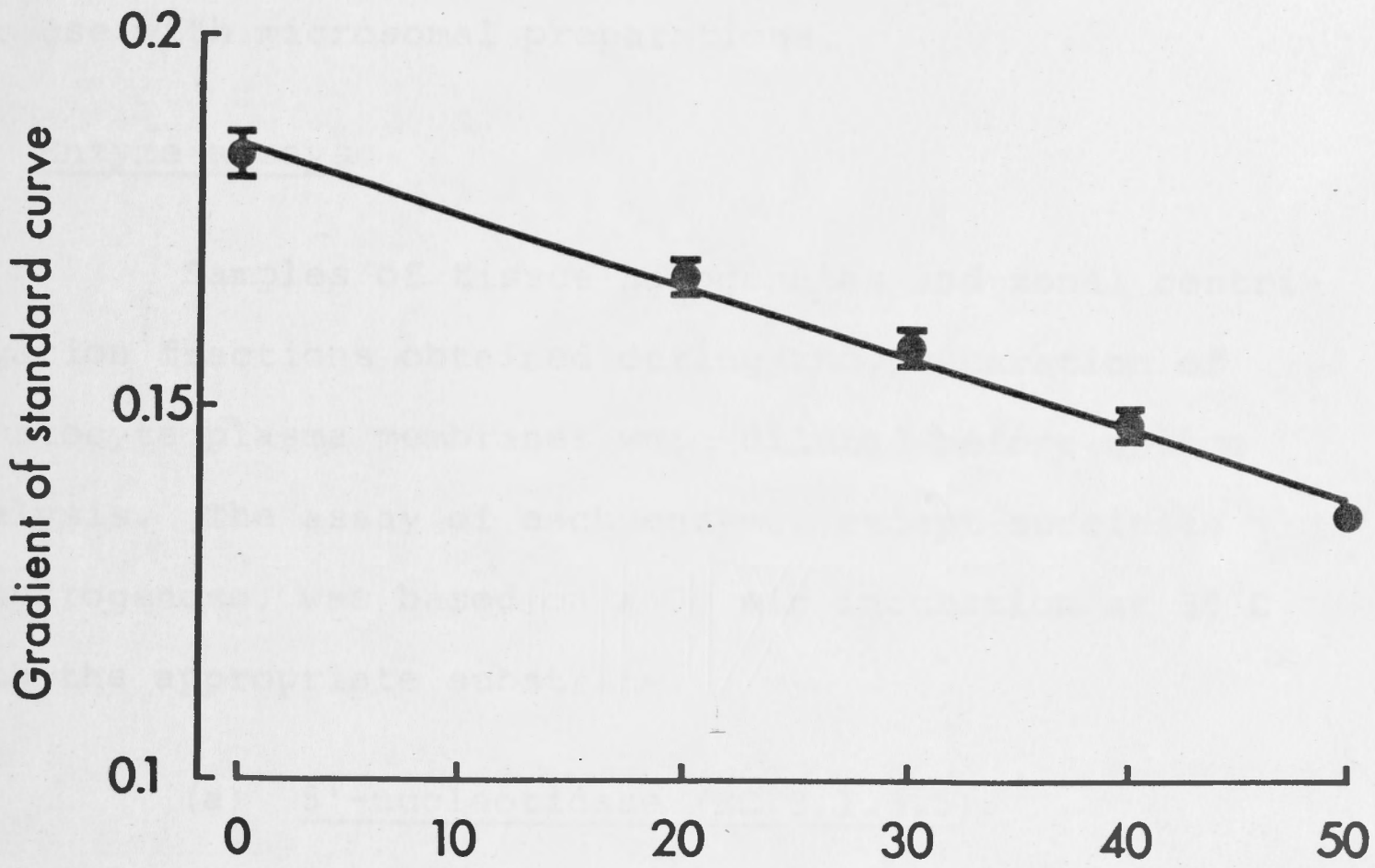


FIGURE 3. *Effect of sucrose on the gradient of the standard curve used for estimation of inorganic phosphate.* Each value is the mean±SEM of 3 determinations. The equation of the regression line is $y = -0.00095x + 0.18494$ ($r = -0.9807$, $P < 0.001$).

The concentrations of protein in microsomal suspensions were determined using the method of Schacterle and Pollack (1973) which was recommended by Albro (1975) for use with microsomal preparations.

6. Enzyme assays

Samples of tissue homogenates and zonal centrifugation fractions obtained during the preparation of hepatocyte plasma membranes were diluted before enzyme analysis. The assay of each enzyme, except succinate dehydrogenase, was based on a 30 min incubation at 30°C with the appropriate substrate.

(a) 5'-nucleotidase (EC 3.1.3.5)

5'-nucleotidase was used as a marker enzyme for plasma membranes. The assay of 5'-nucleotidase involved the release of inorganic phosphate from sodium AMP (Michell and Hawthorne, 1965). Potassium(+)tartrate was added to the incubation medium as an inhibitor of acid phosphatase (El-Aaser and Reid, 1969).

(b) Glucose-6-phosphatase (EC 3.1.3.9)

Glucose-6-phosphatase was used as a marker enzyme for endoplasmic reticulum. The assay of glucose-6-phosphatase involved the release of inorganic phosphate from glucose-6-phosphate disodium salt (Swanson, 1955).

(c) Acid phosphatase (EC 3.1.3.2)

Acid phosphatase was used as a marker enzyme for lysosomes. Acid phosphatase was assayed by measuring the production of *p*-nitrophenol from *p*-nitrophenolphosphate (Bessey *et al.*, 1946) in citrate buffer at pH 5.0.

(d) Succinate dehydrogenase (EC 1.3.99.1)

Succinate dehydrogenase was used as a marker enzyme for mitochondria. Succinate dehydrogenase was assayed by measuring the rate of reduction of 2,6-dichlorophenolindophenol in the presence of sodium succinate (Green *et al.*, 1955) using a recording spectrophotometer (Model SP 8000, Pye Unicam Ltd, Cambridge CBI 2PX, England).

(e) Sucrose interference with enzyme assays

Sucrose has been shown to interfere with the assay of several enzymes (de Duve *et al.*, 1955; El-Aaser *et al.*, 1966b). The results of the 5'-nucleotidase assay were corrected for sucrose interference (Hinton *et al.*, 1969) as the assay was performed directly on zonal centrifugation fractions containing sucrose. Such a correction was not necessary in the assay of the other marker enzymes as the interference from sucrose was negligible due to the large dilution of zonal fractions in the incubation medium.

7. Design of experiments with carcinogen-dosed animals

Weanling female rats and weanling mice of both

sexes were maintained on the low fat diet. When rats had reached a mean age of 32 days and mice had reached a mean age of 70 days they were transferred to the high fat diets containing either polyunsaturated or saturated fat. After a further 28 days each animal received by stomach tube a dose of the carcinogenic polycyclic hydrocarbon DMBA. Rats received 10 mg DMBA dissolved in 0.5 ml sesame oil and mice received 5 mg DMBA in 0.25 ml sesame oil. Control animals received an equal amount of sesame oil. All animals were fed the low fat diet for 2 days before and 2 days after DMBA administration. This temporary change in diet was designed to minimise any possible effects of the dietary fats on the absorption of DMBA from the alimentary tract. Following administration of the DMBA, the diets of half of the animals were interchanged so that animals previously fed the polyunsaturated fat diet were fed the saturated fat diet and vice-versa. The remaining animals were fed the same diet as before the administration of the DMBA.

The male and female mice used to assess the tumorigenic effect of various amounts of DMBA were maintained continually on the low fat diet. At a mean age of 98 days the mice received by stomach tube various amounts of DMBA dissolved in 0.25 ml sesame oil or 0.25 ml sesame oil alone.

All animals were inspected at 9 am and 5 pm on week days and once daily on weekends and holidays. DMBA-dosed rats were palpated for the presence of tumours every 2 weeks. Autopsy was performed when animals had died of natural causes or when they had been killed because death was considered

imminent. The experiment involving DMBA-dosed mice fed the high fat diets was terminated after 90 weeks by killing and autopsying the surviving mice.

8. Statistical analysis of tumour incidences

Differences in the occurrence of tumour-bearing animals were analysed using the method of Peto (1974) which was designed specifically for statistical analysis of tumour incidences in groups of experimental animals. This method draws a distinction between tumours which caused death of the animal or were discovered before the animal died and tumours which were discovered at autopsy when the animal had died of some other cause. Cumulative tumour incidences calculated using Peto's method are not biased by heterogeneous mortality patterns in the different treatment groups. Using this method, the life-time of animals was divided into periods and the groups of animals were compared within each period. The number of animals alive and without tumours at the beginning of each period and the number of animals developing tumours during each period were listed from the experimental record. Assuming the tumorigenic potential of each group of animals to be equal, tables of the number of tumour-bearing animals observed and expected during each period were abstracted. The results of the autopsy of DMBA-dosed mice killed while still apparently healthy were listed on a separate table. The number of these animals with tumours discovered at autopsy during each period and the total number of autopsies performed during each period were listed. The observed and

expected number of tumour-bearing animals were then calculated. The total observed and expected number of tumour-bearing animals which died of natural causes or were killed while apparently healthy were compared by chi-square analysis.

9. Autopsy and histology

Post-mortem examination of animals was performed using the procedure suggested by Arcos *et al.* (1968), for use with animals suspected of bearing tumours, except that the interior of the skull was not examined. Tissues regularly taken for histological examination were; liver, spleen, lungs, mediastinal lymph nodes, kidneys, testes or ovaries, and other tissues which appeared abnormal. Tissues taken at autopsy were fixed in 10% (v/v) formol saline, sectioned and stained with haematoxylin and eosin by the staff of the Histopathology Laboratory in this School. Reticulin, Gram and haemphloxine saffron stains were used by Dr G.C. Hard of the Baker Medical Research Institute, Prahran, Vic 3181, to aid identification of some tumours. Mouse mammary tumours were classified using the histological criteria and nomenclature suggested by Dunn (1959). Mouse liver tumours were classified using the criteria suggested by Butler and Newberne (1975).

10. Pentobarbitone-induced sleeping times of rats

Pentobarbitone-induced sleeping times were determined after each rat had received an intraperitoneal injection of sodium pentobarbitone. The ability of rats to exhibit two righting reflexes within a 30 s period was used to indicate the loss or reappearance of consciousness (Caster

et al., 1970). Sleeping times of rats which had received various amounts of sodium pentobarbitone when they were approximately 60 days of age and were fed the low fat diet are shown in Figure 4. Rats fed either of the high fat diets received injections of sodium pentobarbitone at a concentration of 35 mg/kg body weight.

11. Preparation of microsomes

Rats were killed by exsanguination and their livers perfused *in situ* through the portal vein with ice-cold KCl (1.15% w/v). A representative sample (2 g) of liver was homogenised in 8 ml of the KCl solution using a Potter-Elvehjem teflon-glass homogeniser. The homogenate was centrifuged (centrifuge Model RC2, Ivan Sorvall Inc, Norwalk, Conn 06852, USA) at 9000xg for 20 min to remove the cell debris and mitochondria. After the floating fat layer had been aspirated, the supernatant was decanted and centrifuged at 105,000xg for 60 min in an ultracentrifuge (Model L3-50, Beckman Instruments Inc, Spinco Division, Palo Alto, Ca 94304, USA). The resulting microsomal pellet was resuspended in 8 ml of the KCl solution and centrifuged again at 105,000xg for 60 min. The microsomal pellet was resuspended in 8 ml of 0.1M phosphate buffer (pH 7.0). This procedure produces microsomal fractions with less than 4% contamination by mitochondria (González-Cadavid and Campbell, 1967).

12. Preparation of mouse hepatocyte plasma membranes

(a) Initial fractionation of liver

Mice were killed by decapitation and their livers

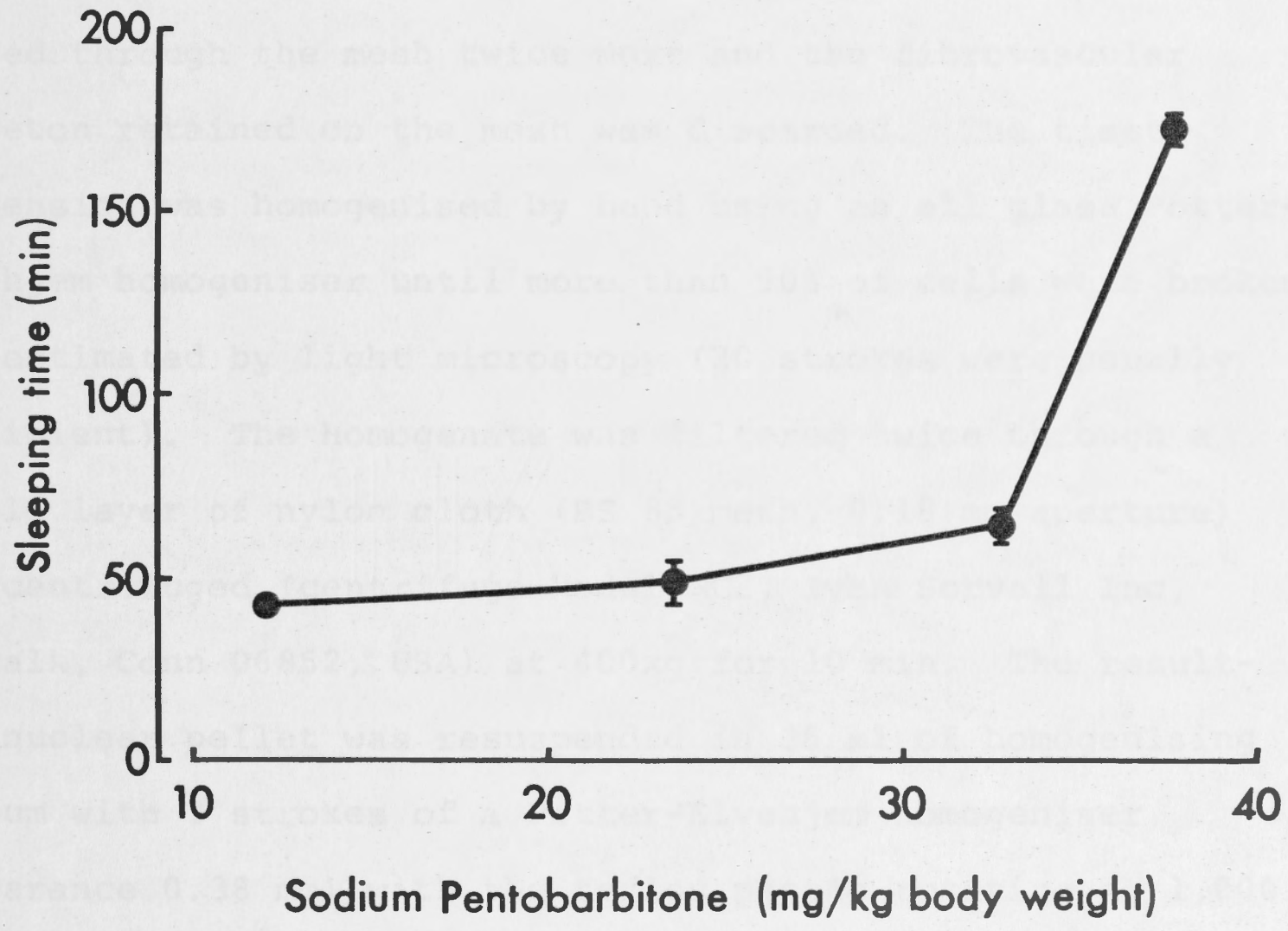


FIGURE 4. *Sleeping times of rats following intraperitoneal injection of sodium pentobarbitone.* Each value is the mean±SEM of determinations on 5 rats.

perfused *in situ* through the portal vein with Ca^{2+} free Hank's solution containing 38mM sodium citrate (Hanks and Wallace, 1949). Following excision, a pooled sample of 4 g of liver from 2 or 3 mice was forced through a stainless steel mesh (BS 14 mesh, 1.18 mm aperture) into 36 ml of 0.25M sucrose. All sucrose solutions used were adjusted to pH 7.6 with sodium bicarbonate. The tissue suspension was then forced through the mesh twice more and the fibrovascular skeleton retained on the mesh was discarded. The tissue suspension was homogenised by hand using an all glass Potter-Elvehjem homogeniser until more than 90% of cells were broken as estimated by light microscopy (20 strokes were usually sufficient). The homogenate was filtered twice through a double layer of nylon cloth (BS 85 mesh, 0.18 mm aperture) and centrifuged (centrifuge Model RC2, Ivan Sorvall Inc, Norwalk, Conn 06852, USA) at 400xg for 10 min. The resulting nuclear pellet was resuspended in 36 ml of homogenising medium with 3 strokes of a Potter-Elvehjem homogeniser (clearance 0.38 mm) with the teflon pestle rotating at 1,000 rev/min. All procedures were carried out at 4°C.

(b) Isolation of plasma membranes by zonal centrifugation

The zonal centrifugation method of Gavard *et al.* (1974) involving a combined rate and isopycnic banding separation, was adapted for use with the zonal rotor available (Model Ti-14, Beckman Instruments Inc Spinco, Division, Palo Alto, Ca 94034, USA). An ultracentrifuge (Model L, Beckman Instruments Inc, Spinco Division, Palo Alto, Ca 94304, USA)

modified for zonal operation and a polystatic pump (Model 2-6100, Buchler Instruments Inc, Fort Lee, NJ 07024, USA) were used. A 440 ml linear density gradient ranging from 20 to 35% w/w sucrose was pumped into the outside of the rotor spinning at 3,500 rev/min. This was followed by 60 ml of 45% w/w sucrose and 150 ml of 55% w/w sucrose to fill the rotor. The prepared homogenate was placed on the gradient and covered by 40 ml of 0.08M sucrose. Centrifugation was continued for 30 min at 3,500 rev/min to achieve a rate separation. Twelve 14 ml samples were then displaced from the core of the rotor and 50 ml of 0.08M sucrose overlay was added to the gradient. Isopycnic banding was achieved by increasing the speed to 23,000 rev/min for 1 h. The entire contents of the rotor were then unloaded as 14 ml fractions by pumping 55% sucrose into the rotor edge. Sucrose densities were determined from sucrose concentrations measured on an Abbe refractometer (Carl Zeiss, Obertroche, GFR). Samples for enzyme analysis either from the zonal separation or from liver homogenates (10% fresh weight by volume) were stored overnight at 4°C. Samples to be analysed for fatty acid composition of lipids were dialysed against distilled water overnight at 4°C, lyophilised using a freeze drying unit (Dynavac High Vacuum Pty Ltd, Burwood, Vic 3125) and stored at -15°C.

13. Electron microscopy

Electron microscopy was used to characterise and to aid the identification of zonal centrifugation fractions containing mouse hepatocyte plasma membranes. Samples of

fractions containing plasma membranes were spread on copper grids (mesh size 150) which had been coated with collodion in iso-amyl acetate (3% w/v), were negatively stained with phosphotungstic acid (1% w/v) and neutralised with 0.1N NaOH. The plasma membranes were examined with an electron microscope (Model EM200, NV Philips, Eindhoven, The Netherlands).

14. Analysis of fatty acids

Gas liquid chromatography was used to identify and quantitate the fatty acids present in the dietary lipids and in the lipids of mouse hepatocyte plasma membranes. Lipids were extracted from aqueous suspensions of feed or plasma membranes with chloroform:methanol (2:1 v/v) (Folch *et al.*, 1957) to which butylated hydroxytoluene (0.005% w/v) had been added to prevent lipid autoxidation (Wren and Szczepanowska 1964). Fatty acids were transesterified using 2,2-dimethoxypropane as described by Mason and Waller (1964). The effectiveness of this procedure was tested and was found to give 97 to 100% recovery of the fatty acids of tripalmitin, tristearin and cholesteryl oleate. The fatty acid methyl esters were analysed using a gas chromatograph (Model 1840, Varian Aerograph, Walnut Creek, Ca 94598, USA) fitted with two stainless steel columns (3 m x 1/8 inch OD) and dual hydrogen flame ionisation detectors. One column was packed with 10% EGSS-X on 80/100 Gas-Chrom Q and the other with 3% JXR on 100/120 Gas-Chrom Q. The Gas-Chrom Q support materials were coated with the EGSS-X and JXR stationary phases as described by Kruppa *et al.* (1967) and the columns were installed and conditioned as described by Vandenheuvel and Court

(1968), except that the conditioning temperatures for the EGSS-X column were adjusted proportionally not to exceed 200°C. Nitrogen was used as a carrier gas at a flow rate of 30 ml/min. The air flow rate was 300 ml/min and the hydrogen flow rate 30 ml/min. The injector, column and detector temperatures were 225, 190 and 225°C, respectively.

Samples were analysed using both columns and the individual fatty acid methyl esters identified by comparing the relative retention times and carbon numbers with those of fatty acid methyl ester standards. Hydrogenation of the unsaturated fatty acid methyl esters (Farquhar *et al.*, 1959) was also used to aid their identification. The identification of some fatty acid methyl esters was checked using a stainless steel column (2 m x 1/8 inch OD) packed with 10% DEGS-PS on 80/100 Supelcoport. In a later experiment fatty acids were analysed using the DEGS-PS and JXR columns. Operating conditions for the DEGS-PS column were the same as for the EGSS-X and JXR columns except that the carrier gas flow was 20 ml/min and the column temperature was 200°C. The DEGS-PS column was conditioned by leaving the column at normal operating temperature and carrier gas flow rate for 3 days.

The relative proportions of fatty acids, except myristic acid, were calculated from the products of the peak heights and retention times of the esters on either the EGSS-X or DEGS-PS column. The proportion of myristic acid was calculated from the relative proportion of methyl myristate to methyl palmitate obtained from data using the JXR column. This procedure was necessary because methyl

myristate and butylated hydroxytoluene had similar retention times on the EGSS-X and DEGS-PS columns.

15. Calculation of relative retention times and carbon numbers of fatty acid methyl esters

Relative retention times can be used to identify fatty acids as their methyl esters on gas chromatograms. However, retention characteristics alter with changes in experimental conditions, e.g. small differences in temperature, gas flow rates, the dead spaces between the column packing and the site of injection and between the packing and the detector. The slight volatility of stationary phases also causes decreases in absolute retention times when the column is used over a long period. This is particularly true for polar polymers customarily used for the separation of unsaturated fatty acid esters. This variability can be reduced by the use of relative apparent retention times (Ackman, 1963) or carbon numbers (Woodford and van Gent, 1960) otherwise known as equivalent chain lengths (Miwa *et al.*, 1960). Both relative apparent retention times and carbon numbers depend on the linear relationship between the carbon number and the logarithm of the retention time of the straight chain saturated acids. A linear relationship also exists between the carbon number and the logarithm of the retention time of unsaturated fatty acids with the same number of double bonds in the same position relative to the terminal methyl group (Ackman, 1963). This means that the difference between the carbon number of unsaturated fatty acids in such a series and the carbon number of their saturated analogues is a constant.

Relative retention times and carbon numbers of fatty acid methyl esters were calculated using a computer. A programme (see Appendix) written for this purpose (West and Rowbotham, 1967) was adapted by Mr H.R. Kinns of this School for use in the Univac 1100-42 computer available.

16. Tumour transplantation

Several female CBA/h mice, each bearing a transplantable mammary adenocarcinoma (number WEHI 8), were kindly given by Dr A.W. Harris of the Walter and Eliza Hall Institute of Medical Research, Parkville, Vic 3052. This tumour originally arose spontaneously in a male C3H mouse. As both CBA and C3H mice bear the H-2^k transplantation allele, mice from either strain can be used as hosts for the tumour.

(a) Passage of tumour

The transplantable tumour was passaged every 3 or 4 weeks into female CBA/h mice. CBA/h mice were used as there was a shortage of C3HA^{vv}fB mice. Tumour-bearing mice were anaesthetised with anaesthetic ether (BP) and killed by cervical dislocation. The tumour was removed, placed in ice-cold HBSS (pH 7.4) and any necrotic tissue removed. Small pieces of tumour tissue were surgically implanted subcutaneously on the dorsal aspect of anaesthetised mice. Surgical instruments were sterilised in Hibitane and the skin swabbed with 70% (v/v) ethanol.

(b) Preparation of tumour cell suspensions

Single cell suspensions of tumour cells were prepared as follows: tumours were placed in ice-cold HBSS and

necrotic tissue removed; one or more tumours were removed from the HBSS and finely minced with scissors; 70 to 75 ml Puck's Saline A containing trypsin (0.25% w/v) was added to the tumour tissue in a conical flask; the flask was stoppered and placed on a magnetic stirrer in a 37°C room for 30 min after which foetal calf serum was added to the cell suspension at a final concentration of 10 to 20% to inactivate the trypsin.

The tumour cell suspension was centrifuged (Mistral Centrifuge Model 6L, Measuring and Scientific Equipment Ltd, London, SW1 England) at 220xg for 3 min. The tumour cells were resuspended in ice-cold Medium 199 and the suspension left to stand in ice for 15 min. The upper portion of the suspension was removed and the cells counted using a haemocytometer. The proportion of viable cells, as estimated by trypan blue exclusion, was regularly between 80 and 90%. The tumour cell suspension was diluted with Medium 199 and injected subcutaneously at an appropriate concentration of viable cells. The C3HA^{vy}fB mice to be inoculated were momentarily anaesthetised with ether to ensure correct positioning of the inoculum in the interscapular region. All instruments, glassware and solutions were previously sterilised in an autoclave.

Inoculated mice were palpated for the presence of tumours every 3 days from the 3rd to the 42nd day after inoculation and then weekly for a further 10 weeks. Tumours were first detected as subcutaneous lumps approximately 2 mm in diameter and the subsequent growth of lumps into large

masses (1 to 2 cm in diameter) was used to confirm the existence of tumours. Histological examination showed that the tumours in the inoculated mice were identical to the original tumour. Using the data from titration experiments, the number of tumour cells required to produce tumours in 50% of the inoculated animals (TD 50) was calculated using the method of Reed and Muench (1938).

RESULTS AND DISCUSSION

1. Influence of dietary fats on pentobarbitone-induced sleeping times and hepatic microsomal cytochrome P-450 in rats

Studies on the mechanisms of action of chemical carcinogens indicate that most of these compounds are not carcinogenic *per se*. They appear instead to be precarcinogens which are converted in the host into carcinogenic electrophilic derivatives termed ultimate carcinogens. The first step in the metabolism of carcinogenic polycyclic hydrocarbons such as 7,12-dimethylbenz(*a*)anthracene (DMBA) is carried out by the mixed function oxygenase enzymes which contain the haemoprotein cytochrome P-450 and are present in the endoplasmic reticulum of cells. Dihydrodiols, glutathione conjugates and phenols have been found as metabolites of aromatic hydrocarbons, while with methylated hydrocarbons, hydroxylation of methyl groups also occurs (Boyland and Sims, 1965, 1967; Sims, 1967; Gentil and Sims, 1971). Another metabolic pathway which has received much attention in recent years is the formation of epoxides, both as a possible step in detoxification (at the M region of the molecule) and in carcinogenesis (at the K region of the molecule) (Berenblum, 1974). The production of epoxy derivatives has been demonstrated by incubating hydrocarbons with liver homogenates or microsomes (Boyland and Sims, 1965; Grover *et al.*, 1971b; Selkirk *et al.*, 1971; Keysell *et al.*, 1973). Such epoxides are more effective alkylating agents than phenolic or dihydroxy derivatives of hydrocarbons (Grover and Sims, 1970; Grover *et al.*, 1971a). Epoxy derivatives of polycyclic aromatic hydrocarbons are also mutagenic (Ames *et al.*, 1972) and can transform normal cells into tumour cells *in vitro* (Grover *et al.*, 1971c; Marquardt *et al.*, 1972).

The activity of the mixed function oxygenase enzymes fluctuates markedly in response to various environmental stimuli (Gelboin, 1972). Dietary fats could be important in determining the activity of these enzymes as they have at least one specific lipid requirement. Phosphatidylcholine is essential for microsomal metabolism because it is necessary for the enzymatic reduction of cytochrome P-450 (Strobel *et al.*, 1970). Increased amounts of dietary fat have also been shown to enhance the activity of the mixed function oxygenase enzymes (Caster *et al.*, 1970; Norred *et al.*, 1971; Norred and Wade, 1972; Patel and Pawar, 1973). In addition, it has been suggested (Marshall and McLean, 1971) that the unsaturation of the dietary lipids and the liability of the lipids to form peroxides may be important in determining mixed function oxygenase activity.

It was therefore of interest to examine the effect of diets containing sunflower-seed oil (polyunsaturated fat diet) or tallow (saturated fat diet) on microsomal metabolism. After rats had been fed either of the diets for 4 weeks pentobarbitone-induced sleeping times were determined. The length of the sleeping time provides a measure of the rate at which the drug is metabolised (Caster *et al.*, 1970; Baumgartner *et al.*, 1974). The concentrations of hepatic microsomal cytochrome P-450 were determined in rats fed either of the diets with or without pentobarbitone pretreatment.

(a) Results

Initial and final body weights of rats fed either the polyunsaturated or the saturated fat diet for 4 weeks are shown in Table 4. There were no statistically significant differences

TABLE 4. Body weights of rats fed either the polyunsaturated or saturated fat diet with or without receiving pentobarbitone in the drinking water.

	Without pentobarbitone				With pentobarbitone	
	Experiment 1		Experiment 2			
	Initial body weight, g	Final body weight, g	Initial body weight, g	Final body weight, g	Initial body weight, g	Final body weight, g
Polyunsaturated fat diet	96.0±2.67	147.7±4.38	114.4±5.07	162.4±4.45	98.5±3.57	148.3±4.33
Saturated fat diet	94.1±2.32	145.9±3.92	115.1±4.78	164.3±5.69	99.1±4.31	153.6±6.05

Each value is the mean±SEM of determinations on 15 animals.

in growth rate, as assessed by body weights, between rats fed either of the diets with or without the addition of pentobarbitone (0.1% w/v) to the drinking water for 2 weeks before death. The consumption of the diets was also similar. In Experiment 2, each rat fed the polyunsaturated fat diet without pentobarbitone consumed 12.0±0.18 g (mean±SEM) feed/day while those fed the saturated fat diet without pentobarbitone consumed 12.2±0.52 g. There were no statistically significant differences between the amounts of pentobarbitone consumed/kg body weight, as estimated from the loss of drinking water from the water bottles, between rats fed either of the diets. Each group of rats consumed between 49 and 52 mg pentobarbitone/kg body weight/day.

In Table 5 are shown the results of Experiment 1, including the concentrations of cytochrome P-450 and microsomal protein, and the liver weights of rats which did not receive pentobarbitone but were fed either of the diets. The mean concentration of cytochrome P-450/kg body weight was greater in rats fed the saturated fat diet than in rats fed the polyunsaturated fat diet. This difference resulted from increases in the concentration of cytochrome P-450/g microsomal protein, the concentration of microsomal protein/g liver and increases in the liver weight/kg body weight. However, none of these differences were statistically significant. This experiment was therefore repeated (Experiment 2) using a second group of rats and the results are also presented in Table 5. The results of experiment 2 confirmed those of experiment 1. Although some loss of microsomes would be expected during the centrifugation of microsomal preparations, the comparisons of the concentrations

TABLE 5. Concentrations of hepatic microsomal cytochrome P-450 in rats fed either the polyunsaturated or saturated fat diet.

	Experiment 1		Experiment 2	
	Polyunsaturated fat diet	Saturated fat diet	Polyunsaturated fat diet	Saturated fat diet
Concentration of cytochrome P-450, μmoles/kg body weight	0.59±0.040	0.66±0.043	0.44±0.041	0.50±0.041
Concentration of cytochrome P-450, μmoles/g microsomal protein	0.74±0.044	0.77±0.042	0.61±0.035	0.63±0.041
Liver weight, g/kg body weight	40.3±0.62	41.1±0.97	35.1±1.07	36.5±1.15
Microsomal protein, mg/g liver	19.8±1.06	21.0±1.59	20.2±1.13	21.7±0.87

Each value is the mean±SEM of determinations on 15 animals.

of cytochrome P-450 and the concentrations of microsomal protein are valid as relatively large numbers of animals were used and similar losses would be expected in each sample. Similar comparisons of concentrations of cytochrome P-450 have also been made by other workers (Marshall and McLean, 1971; Norred and Wade, 1972).

Pentobarbitone-induced sleeping times of rats which did not receive pentobarbitone in the drinking water and were fed either the polyunsaturated or the saturated fat diet are shown in Table 6. These measurements were made on animals which were not used for the determination of concentrations of cytochrome P-450. Rats fed the polyunsaturated fat diet slept for longer times than rats fed the saturated fat diet. This difference was statistically significant (Student's t-test, $P < 0.05$).

The concentrations of cytochrome P-450 and microsomal protein, and the liver weights of rats which received pentobarbitone in the drinking water and were fed either of the diets are shown in Table 7. The mean concentration of cytochrome P-450/kg body weight was greater in rats fed the saturated fat diet than in rats fed the polyunsaturated fat diet. This difference, which was statistically significant (Student's t-test, $P < 0.01$), resulted from increases in the concentration of cytochrome P-450/g microsomal protein and in the liver weight/kg body weight. The addition of pentobarbitone to the drinking water of these rats caused 2 to 3 fold increases in the concentrations of cytochrome P-450/kg body weight. This increase was slightly greater in rats fed the saturated fat diet than in rats fed the polyunsaturated fat diet.

TABLE 6. Pentobarbitone-induced sleeping times of rats fed either the polyunsaturated or saturated fat diet.

	Polyunsaturated fat diet	Saturated fat diet
Sleeping time, min	63.0±4.04	48.4±2.71[a]

Each value is the mean±SEM of determinations on 23 animals. Comparison by Student t-test: [a]$P < 0.05$.

TABLE 7. Concentrations of hepatic microsomal cytochrome P-450 in rats receiving pentobarbitone in the drinking water and fed either the polyunsaturated or saturated fat diet.

	Polyunsaturated fat diet	Saturated fat diet
Concentration of cytochrome P-450, µmoles/kg body weight	1.27±0.039	1.63±0.086[a]
Concentration of cytochrome P-450, µmoles/g microsomal protein	1.43±0.040	1.82±0.069[a]
Liver weight, g/kg body weight	42.9±0.70	45.4±0.89[b]
Microsomal protein, mg/g liver	20.9±0.65	19.6±0.69

Each value is the mean±SEM of determinations on 15 animals. Comparisons by Student t-test: [a]$P < 0.01$, [b]$P < 0.05$.

(b) Discussion

These results show that rats fed the saturated fat diet had greater concentrations of hepatic microsomal cytochrome P-450 than rats fed the polyunsaturated fat diet. Rats with greater concentrations of cytochrome P-450 slept for shorter times after injection with pentobarbitone. This was expected as it is known that increases in drug metabolism are accompanied by increases in the concentration of cytochrome P-450 (Remmer and Merker, 1965; Guarino *et al.*, 1969).

Other workers (Century and Horwitt, 1968; Marshall and McLean, 1971; Century, 1973) have shown that the rate of microsomal metabolism and the concentration of hepatic microsomal cytochrome P-450 are greater in phenobarbitone-treated rats fed diets containing polyunsaturated fats rather than more saturated fats. It is possible that either the degree of unsaturation of the dietary lipids or their propensity to form lipid peroxides was responsible for the results of these experiments (Marshall and McLean, 1971). As particular care was taken to reduce lipid peroxidation in the diets used in the present experiments to a minimum, it is possible that greater amounts of lipid peroxides in the diets used by other workers may be responsible for the discrepancy between their results and the results presented here.

Attempts to rationalise the interaction of a wide range of substrates with a common cytochrome P-450 have led to the discovery of a multiplicity of components of cytochrome P-450 and the existence of substrate specificity (Wickramasinghe, 1975). As dietary factors may preferentially

influence one or more forms of the cytochrome this topic requires further investigation. The effect of dietary fats on several mono-oxygenase activities, such as the conversion of aniline to *p*-aminophenol and laurate to ω-hydroxylaurate, and on NADPH-cytochrome P-450 reductase would be of interest.

The stimulation of microsomal metabolism by certain drugs, including barbiturates, is a well known phenomenon. Administration of phenobarbitone to rats causes induction of hepatic microsomal mixed function oxygenase activity, but does not increase the activity of other microsomal enzyme systems (Jansson and Schenkman, 1975). Liver microsomes from rats pre-treated with phenobarbitone have an increased ability to metabolise a range of compounds including other barbiturates, aminopyrine, phenylbutazone, zoxazolamine and carcinogenic azo dyes and polycyclic hydrocarbons (Conney *et al.*, 1960). Morphological changes in the liver cell, principally an increase in the quantity of smooth endoplasmic reticulum, also accompany the induction of enzyme activity (Conney, 1967). In the present experiments the effect of dietary fats on the concentrations of cytochrome P-450 and microsomal protein was studied in rats receiving pentobarbitone as it is known that chemical carcinogens, such as polycyclic hydrocarbons, stimulate their own metabolism (Conney *et al.*, 1960; Jellinck and Goudy, 1967; Levin and Conney, 1967).

The activity of the microsomal enzymes may be an important factor in human carcinogenesis as it is thought that as many as 90% of cancers seen in man are caused by environmental factors, mostly chemicals (Maugh, 1974). Many chemical

carcinogens are converted into active carcinogenic derivatives by these enzymes. In addition to producing more active derivatives these enzymes also produce less active derivatives (Farber, 1973). The balance between the activating and de-activating reactions is important in determining the toxic and carcinogenic effects of many compounds. The stimulation of microsomal metabolism by polycyclic hydrocarbons or barbiturates has been shown to protect against the toxic effects of DMBA. This is associated with decreased concentrations of DMBA and DMBA metabolites in the tissues, and increased ring hydroxylation at the expense of side chain oxidation (Sims, 1966; Jellinck and Goudy, 1967; Levin and Conney, 1967; Sims and Grover, 1967). Ring hydroxylation is thought to be a process of deactivation allowing increased formation of water-soluble compounds. A considerable number of *in vivo* experiments have shown that chemical carcinogenesis is also inhibited by the induction of microsomal enzymes (Wattenberg, 1975). This topic has been discussed more extensively in the Introduction. The decreased microsomal enzyme activity of rats fed the polyunsaturated fat diet, compared to rats fed the saturated fat diet, may therefore have enhanced their susceptibility to the action of chemical carcinogens.

However, it does not necessarily follow that increased microsomal metabolism will be associated with an inhibition of carcinogenesis induced by a long exposure to small concentrations of carcinogens. Low levels of carcinogen may not saturate the binding sites for the ultimate carcinogen and increased microsomal enzyme activity may therefore enhance the carcinogenic effect of the compound. Although increased

deactivation, as well as increased activation, of the carcinogen may result from the induction of microsomal enzymes, once enough of the ultimate carcinogen has accumulated carcinogenesis will be initiated. This is consistent with the finding of Kellerman *et al.* (1973) that the susceptibility of people to lung cancer, which is considered to be induced at least partly by chemicals, is associated with greater inducible aryl hydrocarbon hydroxylase activities.

2. Effect of dietary fats on 7,12-dimethylbenz(*a*)anthracene-induced tumours in rats

Several groups of workers have shown that the susceptibility of rats and mice to develop mammary and skin tumours is enhanced by an increased proportion of fat in the diet (Tannenbaum and Silverstone, 1957; Carroll *et al.*, 1968; Carroll and Khor, 1975). This effect of dietary fat is due to a specific action of the fat rather than to a calorific effect (Tannenbaum, 1945; Tannenbaum and Silverstone, 1957). Studies by Carroll and his colleagues on mammary carcinogenesis induced by the polycyclic hydrocarbon 7,12-dimethylbenz(*a*)anthracene (DMBA) have suggested that the nature as well as the amount of dietary fat may influence the incidence of cancer. Thus Gammal *et al.* (1967) found enhanced development of DMBA-induced tumours in rats fed a semisynthetic diet containing corn oil (an unsaturated oil) rather than coconut oil (a more saturated oil).

In the present study the effect of the polyunsaturated and saturated fat diets on the incidence of DMBA-induced tumours in rats was examined. In order to identify the stage of carcinogenesis which might be influenced by dietary fat, some of the rats received different diets before and after DMBA administration.

(a) Results

There were no meaningful differences in growth rate, as assessed by body weights, between control and DMBA-dosed rats fed the polyunsaturated fat diet or the saturated fat diet (Figure 5). When rats were fed a different diet after DMBA administration the ensuing growth rate was not altered

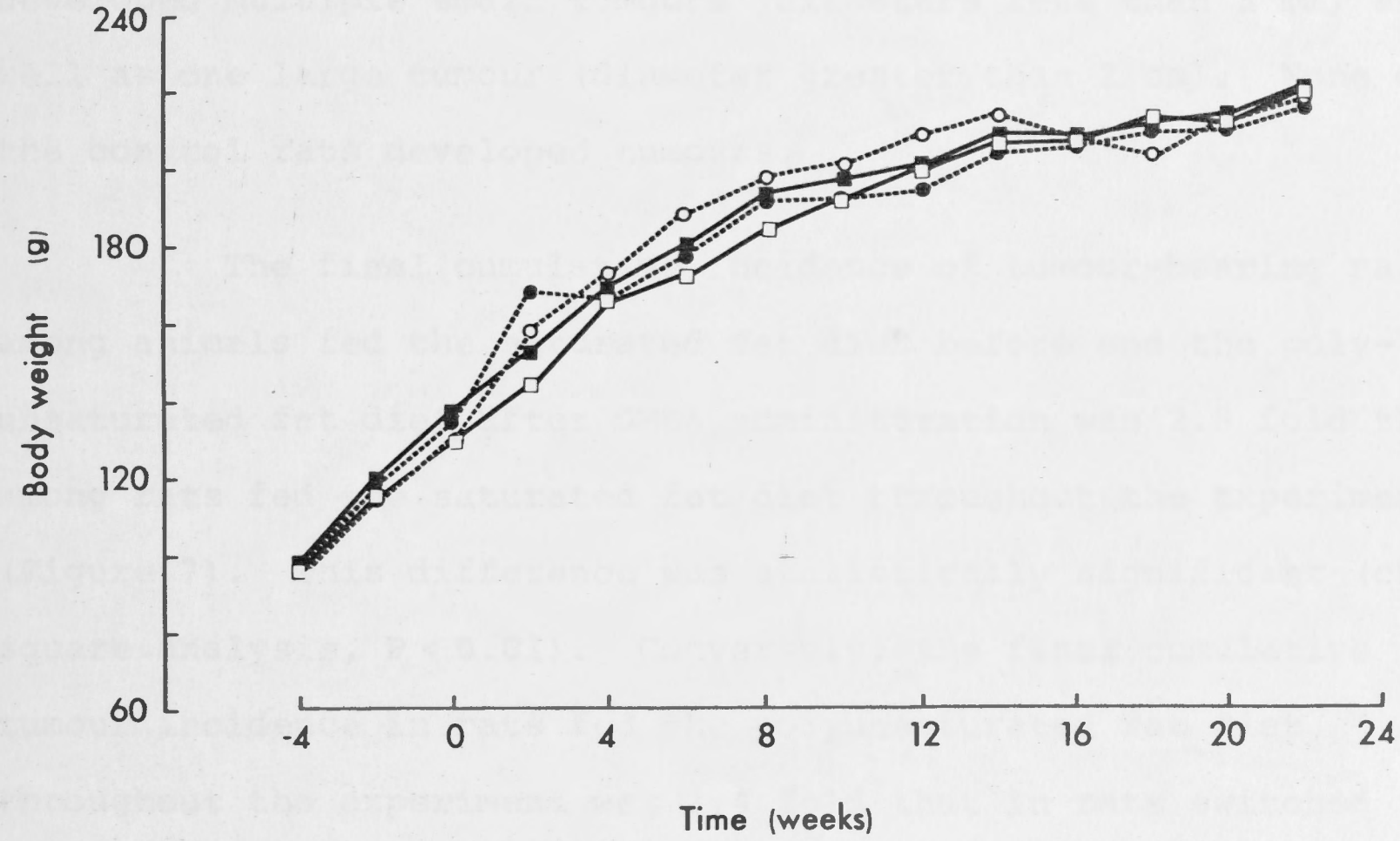


FIGURE 5. *Mean body weights of DMBA-dosed rats fed the polyunsaturated fat diet (■——■) or the saturated fat diet (□——□) throughout the experiment, and control rats fed the polyunsaturated fat diet (●·····●) or the saturated fat diet (○·····○) throughout the experiment.* The rats which received DMBA were dosed at week zero.

by the change of diet (Figure 6).

The number of rats which developed tumours and the total number of rats in each experimental group are shown in Table 8. Most of the tumour-bearing rats developed one tumour each. Three rats developed two tumours each and one rat developed multiple small tumours (diameters less than 3 mm) as well as one large tumour (diameter greater than 2 cm). None of the control rats developed tumours.

The final cumulative incidence of tumour-bearing rats among animals fed the saturated fat diet before and the polyunsaturated fat diet after DMBA administration was 2.8 fold that among rats fed the saturated fat diet throughout the experiment (Figure 7). This difference was statistically significant (chi-square analysis, $P < 0.01$). Conversely, the final cumulative tumour incidence in rats fed the polyunsaturated fat diet throughout the experiment was 3.4 fold that in rats switched to the saturated fat diet after DMBA administration (Figure 8). This difference was also statistically significant (chi-square analysis, $P < 0.025$). The cumulative tumour incidence in rats fed the polyunsaturated fat diet throughout the experiment was consistently greater than that in rats fed the saturated fat diet throughout the experiment (Figure 9). However, this difference was not statistically significant (chi-square analysis, $P < 0.1$). There were also no statistically significant differences between the tumour incidences in rats fed different diets before DMBA administration and the same diet after DMBA administration.

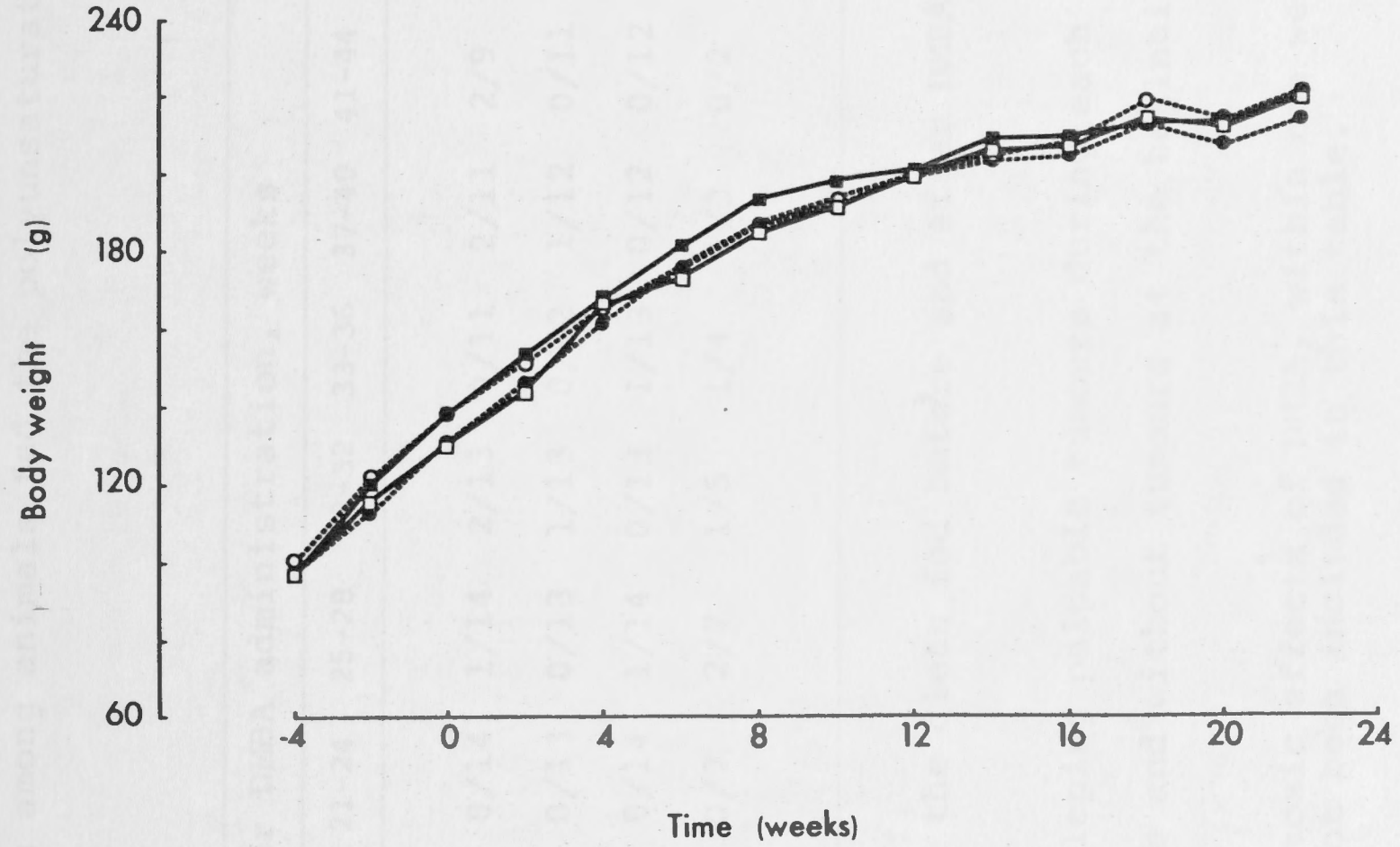


FIGURE 6. *Mean body weights of DMBA-dosed rats fed the polyunsaturated fat diet (■——■) or the saturated fat diet (□——□) throughout the experiment, and DMBA-dosed rats fed the saturated fat diet before and the polyunsaturated fat diet after DMBA administration (●·····●) or vice-versa (○·····○).* The rats which received DMBA were dosed at week zero.

TABLE 8. Number of tumour-bearing rats among animals fed the polyunsaturated fat diet (P) or the saturated fat diet (S).

Diet	Time after DMBA administration, weeks													
	1-4	5-8	9-12	13-16	17-20	21-24	25-28	29-32	33-36	37-40	41-44	45-48	49-52	53-56
PP[a]	0/17[bc]	0/17	3/17	0/14	0/14	0/14	1/14	2/13	0/11	2/11	2/9	2/7	1/2	0/1[d]
SS	0/17	0/17	0/17	1/14	0/13	0/13	0/13	1/13	0/12	1/12	0/11	2/11	4/9	1/5
PS	0/15	0/15	0/15	0/15	1/15	0/14	1/14	0/13	1/13	0/12	0/12	1/12	1/11	1/10
SP	0/13	0/13	1/13	3/11	0/8	0/7	2/7	1/5	1/4	1/3	0/2	0/2	1/2	1/1

[a] The first and second letters refer to the diets fed before and after DMBA administration, respectively.

[b] x/y: x is the number of animals developing palpable tumours during each 4 week period.

y is the number of animals alive and without tumours at the beginning of each 4 week period.

[c] Some animals died, presumably of the toxic effects of DMBA, within one week of its administration. These animals have not been included in this table.

[d] By week 56 all animals had died of natural causes or had been killed because of their deteriorating condition.

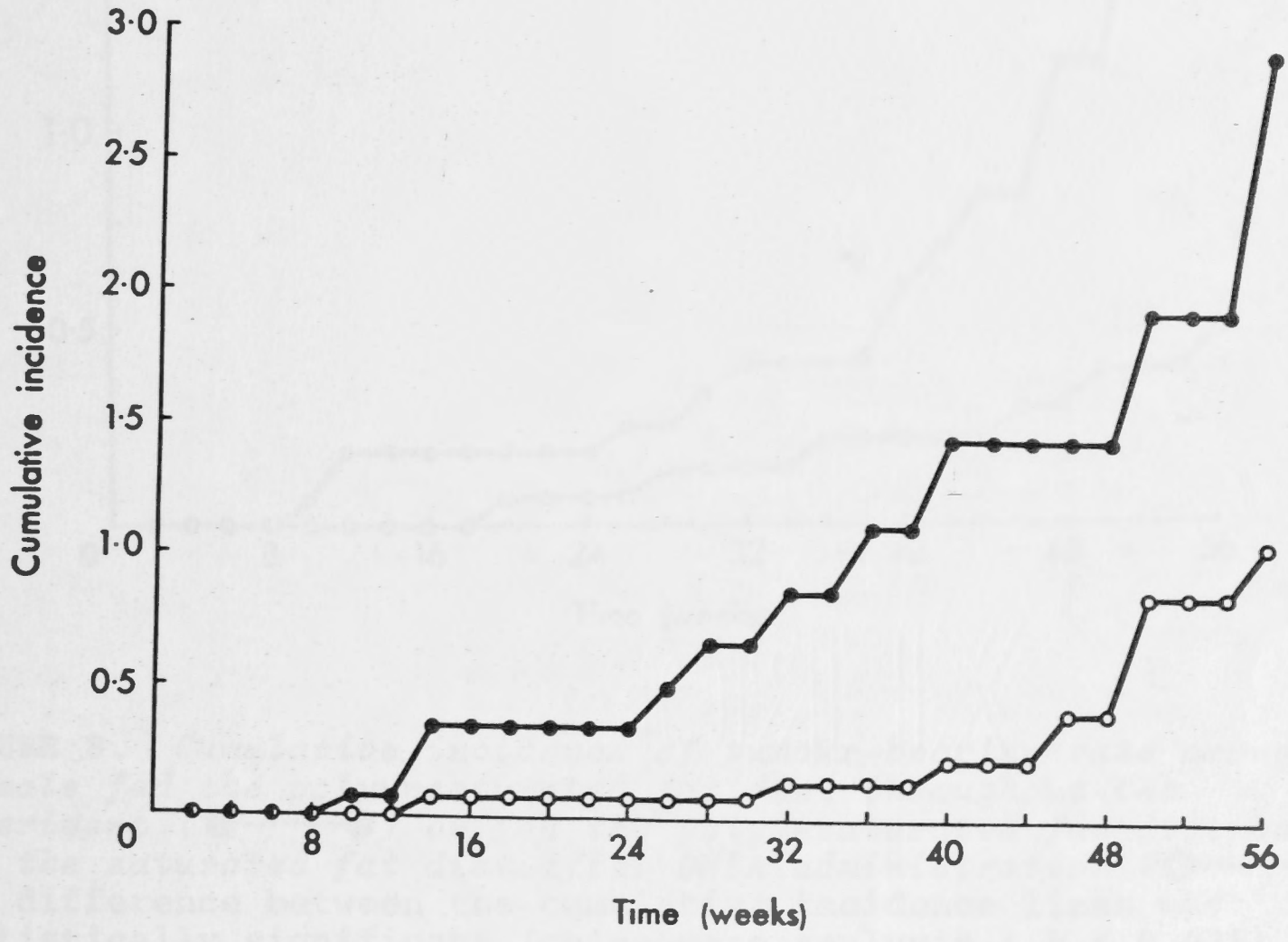


FIGURE 7. *Cumulative incidence of tumour-bearing rats among animals fed the saturated fat diet throughout the experiment (○——○) or fed the saturated fat diet before and the poly-unsaturated fat diet after DMBA administration (●——●).* The difference between the cumulative incidence lines was statistically significant (chi-square analysis : $P < 0.01$). In all cumulative tumour incidence graphs in this thesis the animals which received DMBA were dosed at week zero.

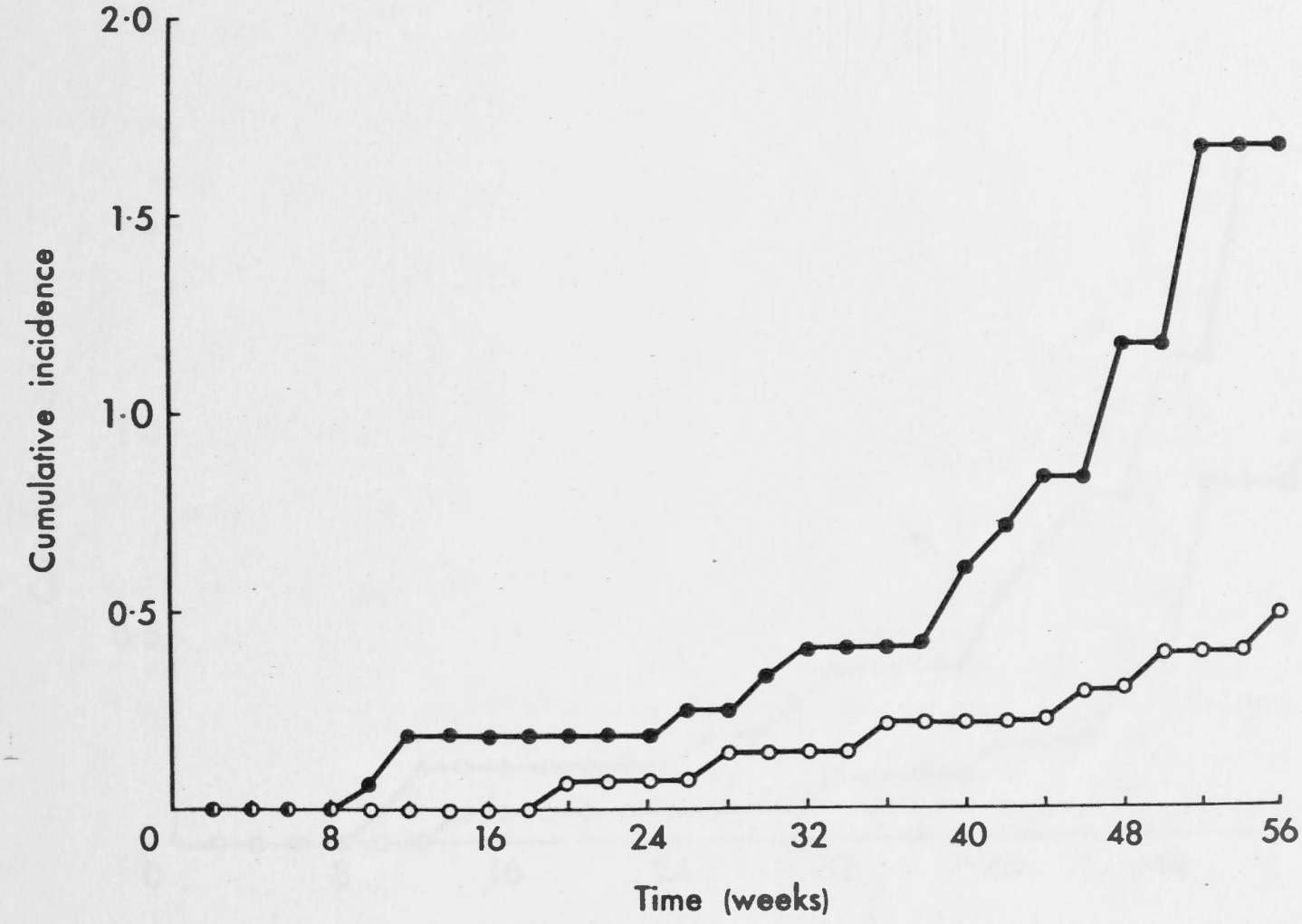


FIGURE 8. *Cumulative incidence of tumour-bearing rats among animals fed the polyunsaturated fat diet throughout the experiment (●——●) or fed the polyunsaturated fat diet before and the saturated fat diet after DMBA administration (○——○).* The difference between the cumulative incidence lines was statistically significant (chi-square analysis : $P < 0.025$).

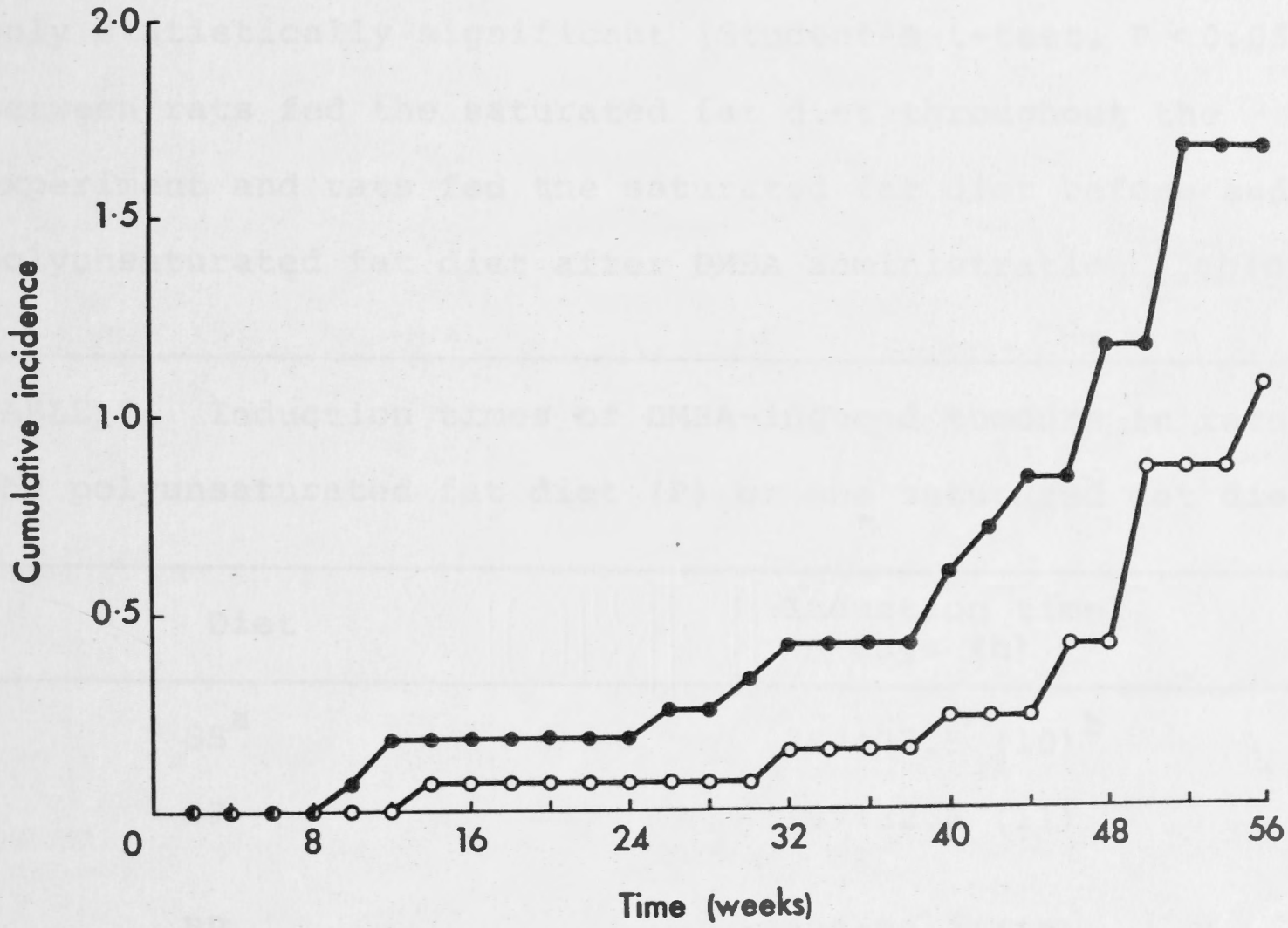


FIGURE 9. *Cumulative incidence of tumour-bearing rats among animals fed the polyunsaturated fat diet (●——●) or the saturated fat diet (○——○) throughout the experiment.* The difference between the cumulative incidence lines was not statistically significant.

The mean times elapsed before palpable tumours developed (mean induction times) were less when rats were fed the polyunsaturated fat diet, rather than the saturated fat diet, after DMBA administration. However, this difference was only statistically significant (Student's t-test, $P < 0.05$) between rats fed the saturated fat diet throughout the experiment and rats fed the saturated fat diet before and the polyunsaturated fat diet after DMBA administration (Table 9).

TABLE 9. Induction times of DMBA-induced tumours in rats fed the polyunsaturated fat diet (P) or the saturated fat diet (S).

Diet	Induction time days (n)
SS[a]	294±27.5 (10)[b]
SP	197±32.5 (11)
PP	228±28.3 (13)
PS	272±37.1 (6)
SS	294±27.5 (10)
PP	228±28.3 (13)
PP + SP	213±21.2 (24)[b]
SS + PS	285±21.5 (16)
PP + PS	242±22.6 (19)
SS + SP	243±23.6 (21)

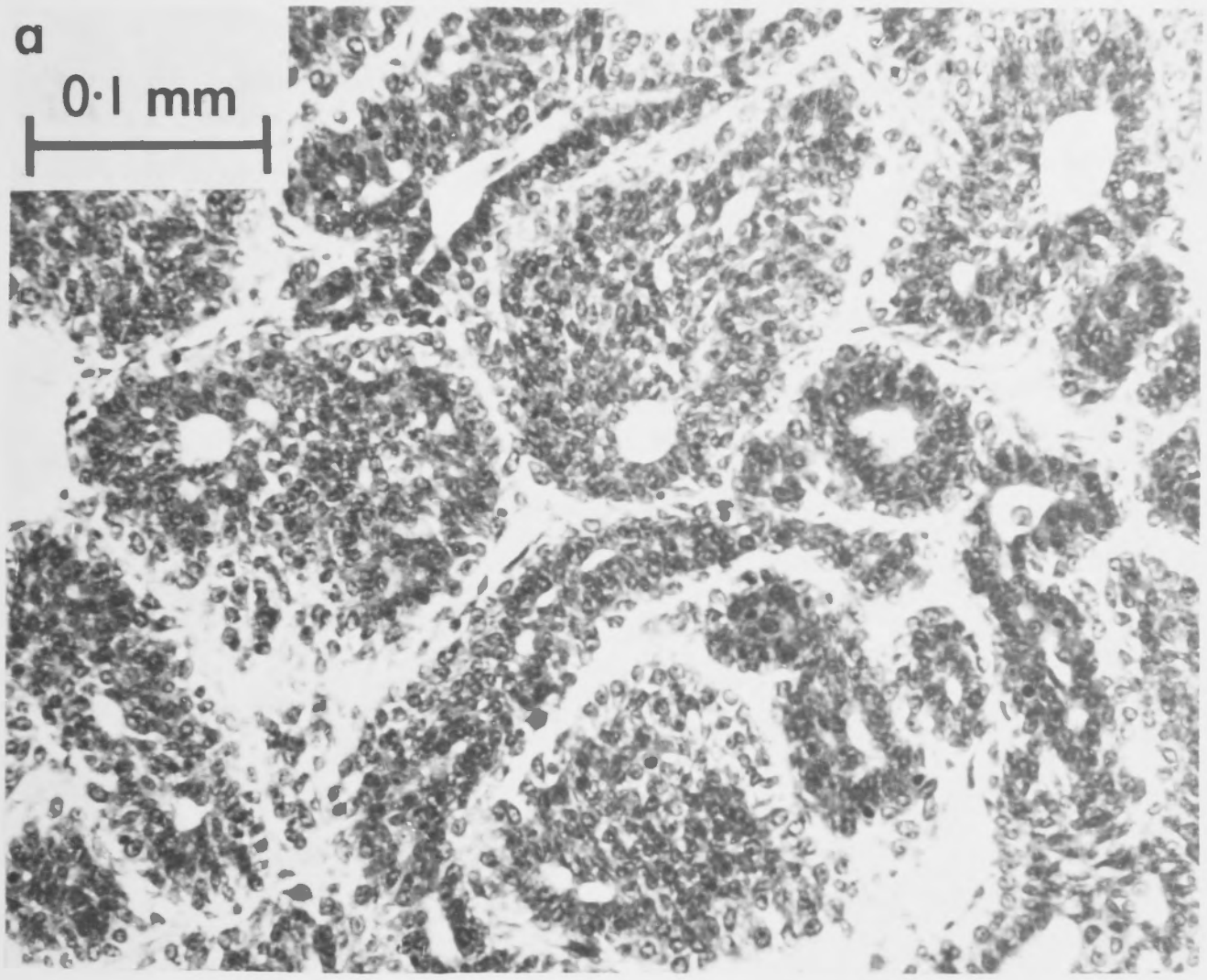
Each value is the mean±SEM.

[a]See footnote a of Table 8.

[b]Comparison by Student's t-test with group immediately below: $P<0.05$.

A comparison of the mean induction times of tumours among all rats fed either diet after DMBA administration, regardless of the diet fed previously, revealed that the mean induction time was less when rats were fed the polyunsaturated fat diet, rather than the saturated fat diet, after DMBA administration. This difference was statistically significant (Student's t-test, $P < 0.05$). This comparison is only valid if a similar comparison of induction times is made between rats fed different diets before DMBA administration regardless of the diet fed after DMBA administration. In this case, the difference between the mean induction times was not statistically significant.

The majority of tumours were either benign fibro-epithelial tumours or malignant epithelial tumours of the mammary gland. One animal developed a squamous cell carcinoma of the skin and another developed a squamous cell papilloma of the skin. Photomicrographs of the most common types of tumours found are shown in Figure 10. Malignant tumours developed in 64% of the tumour-bearing rats fed the polyunsaturated fat diet throughout the experiment. When rats were switched to the saturated fat diet after DMBA administration only 40% of the tumour-bearing rats developed malignant tumours. Malignant tumours developed in 40% of the tumour-bearing rats fed the saturated fat throughout the experiment, but this figure rose to 80% when rats were switched to the polyunsaturated fat diet after DMBA administration. Four rats which had tumours at least 2 cm in diameter died unexpectedly and were eaten by other rats before autopsies could be performed. Two of these rats had been fed the polyunsaturated fat diet throughout the



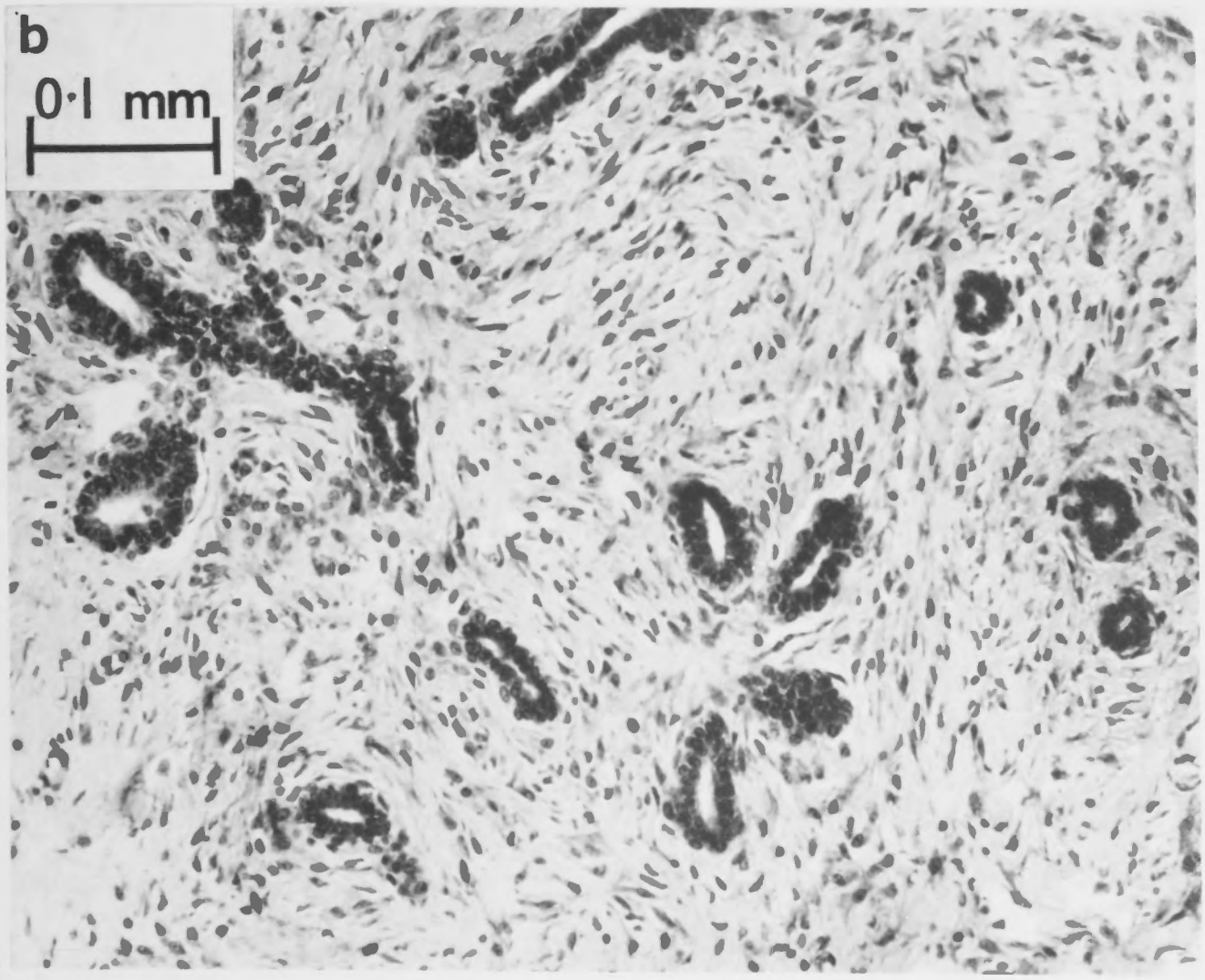


FIGURE 10. *Photomicrographs of the most common types of tumours in DMBA-dosed rats.* a. Example of a malignant epithelial mammary tumour - adenocarcinoma. Haematoxylin and eosin; magnification 245X. b. Example of a benign fibroepithelial mammary tumour - pericanicular fibroadenoma. Haematoxylin and eosin; magnification 195X. Courtesy of Dr G.C. Hard.

experiment, one had been switched to the polyunsaturated fat diet after DMBA administration and the other to the saturated fat diet after DMBA administration.

(b) Discussion

The results of this experiment show that the incidence of tumour-bearing rats and the proportion of such rats which developed malignant tumours were greater among those fed the polyunsaturated fat diet than among those fed the saturated fat diet. The mean induction time of tumours in rats fed the polyunsaturated fat diet was also less than in rats fed the saturated fat diet. This enhancement of carcinogenesis by the polyunsaturated fat diet was exerted only when the diet was fed after DMBA administration. However, the lack of a statistically significant difference between the tumour incidences when rats were fed either diet throughout the experiment and the presence of statistically significant differences when rats were fed a different diet after DMBA administration indicates that the change in diet could have been of importance in determining the tumour incidence. The observed differences in tumorigenesis are unlikely to be due to differences in the absorption of DMBA from the alimentary tract as all animals were transferred to a low fat diet for 2 days before and 2 days after the administration of DMBA.

These results support those of Gammal *et al.* (1967) who showed that the incidence of palpable tumours was greater among DMBA-dosed rats fed a semisynthetic high corn oil diet, rather than an isocaloric diet containing coconut oil. These authors did not find statistically significant differences

between tumour incidences based on the total number of tumours discovered at autopsy 4 months after DMBA administration. As tumours may occasionally regress tumour-bearing rats in the experiments reported in this thesis were allowed to die of natural causes or to survive until their general appearance had indicated that death was imminent. The death or imminent death of an animal was regarded as a more meaningful measure of the incidence of cancer in the groups of experimental animals than the number of tumours discovered at autopsy of animals killed while still apparently healthy.

As discussed in the Introduction to this thesis the carcinogenic process can be divided into two distinct stages, commonly referred to as those of initiation and promotion. The first stage is thought to involve the rapid and irreversible conversion of normal cells into neoplastic cells by interaction with a carcinogenic agent. The second stage involves the cellular proliferation of neoplastic cells to form an established tumour. Since the enhancement of carcinogenesis was observed only when the polyunsaturated fat diet was fed to rats after DMBA administration, it is likely that the effect of this diet was upon the survival and proliferation of tumour cells, rather than the initial event of neoplastic transformation. This conclusion is consistent with the finding that the higher incidence of palpable tumours observed by Gammal *et al*. (1967) in rats fed a high corn oil diet, was not the result of a higher concentration of DMBA in the tissues or a longer exposure of the tissues to the carcinogen (Gammal *et al*., 1968). In addition, Carroll and Khor (1975) have found that the tumour yield in rats dosed with DMBA was enhanced if the rats were

transferred from a low corn oil diet to a high corn oil diet 1 or 2 weeks after giving the DMBA. If the transfer was delayed for 4 weeks little or no enhancement was seen. From the results of the experiment reported here and those of Carroll's research group it appears that this is a crucial period for the development of tumours from neoplastic cells.

One physiological system which may influence the promotional stage of carcinogenesis is the immune system. Cell-mediated immune reactions can be generated in response to tumour-associated antigens and are generally recognised to be of consequence to the potential tumour-bearing host. It is therefore possible that the enhancement of tumorigenesis by dietary polyunsaturated fat may have an underlying immunological basis. This hypothesis is supported by reports that polyunsaturated fatty acids inhibit the *in vitro* antigen-induced response of lymphocytes (Mertin *et al.*, 1974; Offner and Clausen, 1974; Mertin and Hughes, 1975). Polyunsaturated fatty acids have also been shown to prolong the survival of skin grafts in rodents (Mertin, 1974; Ring *et al.*, 1974; Mertin, 1976) and to be beneficial as an adjunct to immunosuppressive therapy following human renal transplantation (Uldall *et al.*, 1975). The reports of an improved clinical course of multiple sclerosis patients fed linoleate (Millar *et al.*, 1973), a higher incidence of cancer among atherosclerotic patients fed a diet containing polyunsaturated fat (Pearce and Dayton, 1971) and an increased susceptibility to allergic encephalomyelitis of rats bred and raised on a diet deficient in polyunsaturated fatty acids (Clausen and Møller, 1967) may

also be explicable in terms of a suppression of immune function by polyunsaturated fatty acids.

It is possible that the immuno-inhibitory action of these fatty acids is mediated through prostaglandins, as prostaglandins E_1 and E_2 (PGE_1 and PGE_2, respectively) are synthesised by plasma membrane-bound prostaglandin synthetase from linoleic and arachidonic acids, respectively (Ramwell *et al.*, 1968). PGE_1 and PGE_2 inhibit the *in vitro* antigen-induced lymphocyte response (Offner and Clausen, 1974), PGE_1 prolongs homograft survival in mice (Quagliata *et al.*, 1973), and the prostaglandin-stimulated synthesis of cyclic 3',5'-adenosine monophosphate (de Boer *et al.*, 1973) regulates the expression of immediate and delayed hypersensitivity (Henney *et al.*, 1972; Lichtenstein *et al.*, 1972; Strom *et al.*, 1973).

As increases in the dietary intake of linoleic acid may lead to increases in the synthesis of PGE_1 (Thomasson, 1969; Vergroesen and Gottenbos, 1975) it is possible that increased concentrations of PGE_1 may have been partly or wholly responsible for the enhancement of tumorigenesis observed in rats fed the polyunsaturated fat diet (60% of fatty acids being linoleic acid). In this context, it is interesting to note that DMBA-induced mammary tumours in rats contain greater concentrations of prostaglandin than do normal rat mammary glands (Tan *et al.*, 1974). Inhibitors of prostaglandin synthesis have been shown to block the immuno-suppression caused by other types of tumours and to retard the growth of these tumours (Plescia *et al.*, 1975).

It is also possible that the feeding of either the polyunsaturated or saturated fat diet to the animals may have altered the endocrine environment which is known to be important in determining the growth of some tumours (McGuire *et al.*, 1974). High fat diets which promote tumorigenesis (Carroll and Khor, 1970; Chan and Cohen, 1974) also raise the concentration of serum prolactin in rats at proestrus-oestrus (Chan *et al.*, 1975). The ratio of the concentrations of prolactin to oestrogen in the serum has been suggested to regulate the growth of rat mammary tumours (Chan and Cohen, 1975).

While the different fatty acid compositions of the dietary fats could have been responsible for the enhancement of carcinogenesis observed in rats fed the polyunsaturated fat diet, it is also possible that other constituents of the dietary fats may have been important. These could have included cholesterol in the saturated fat diet, plant sterols in the polyunsaturated fat diet and different anti-oxidants, including tocopherols, in the diets. Sunflower-seed oil contains large amounts of tocopherols with reported concentrations varying from about 270 to 600µg/g (Herting and Drury, 1963). These differences in concentration may have resulted from differences in the age and source of the oils, the extent to which the oils were processed and the methods used to determine the concentration of tocopherols (Herting and Drury, 1963). In contrast tallow contains negligible amounts of tocopherols (Ames, 1972).

The anti-oxidants butylated hydroxyanisole, butylated hydroxytoluene and ethoxyquin have been shown to inhibit

the action of carcinogens under diverse experimental conditions (Frankfurt *et al.*, 1967; Wattenberg, 1972a, 1972b; Ulland *et al.*, 1973; Wattenberg, 1973). Several other anti-oxidants with different structural characteristics also inhibit chemical-induced carcinogenesis (Wattenberg, 1974). There have been contradictory reports on the effect of tocopherols on chemical-induced carcinogenesis when different carcinogens or routes of administration of the anti-oxidant were used (Haber and Wissler, 1962; Wattenberg, 1972a; Smith and Kenyon, 1973). However, tocopherols incorporated in the diet have been shown to inhibit the induction of mammary tumours in DMBA-dosed rats (Harman, 1969). It is therefore unlikely that anti-oxidants were important in determining the increased tumour incidence in rats fed the polyunsaturated fat diet, as this diet contained greater amounts of anti-oxidants than the saturated fat diet.

As reported in Section 1, rats fed the polyunsaturated fat diet for 4 weeks had decreased rates of drug metabolism and decreased concentrations of hepatic microsomal cytochrome P-450 compared with rats fed the saturated fat diet for 4 weeks. These changes would be expected to enhance the susceptibility of rats fed the polyunsaturated fat diet to develop DMBA-induced tumours (Wattenberg, 1975). However, the decreased activity of the microsomal mixed function oxygenase enzymes was probably not important in determining the tumour incidence in the DMBA-dosed rats reported here as only the diet fed after DMBA administration influenced the tumour incidence.

3. Effect of dietary fats on 7,12-dimethylbenz(*a*)anthracene-induced tumours in mice

The experiment described in Section 2 showed that carcinogenesis was enhanced when female rats were fed the polyunsaturated fat diet. This effect appeared to be exerted during the promotional stage of carcinogenesis. In order to substantiate this effect a similar experiment was carried out using male and female C3HA^{vy}fB mice. As the amount of DMBA required to induce tumours in these animals was not known, male and female mice fed the low fat diet received various amounts of DMBA in multiple and single intragastric doses.

(a) Results

(i) Tumours in mice dosed with various amounts of carcinogen and fed a low fat diet

The multiple doses of DMBA which were given to mice were extremely toxic. After receiving the last of 4 weekly doses of 5 mg DMBA only 2 male mice and 3 female mice out of groups of 20 mice survived for 3 weeks. When mice received 3 weekly doses of 5 mg DMBA 7 male mice and 6 female mice out of groups of 20 mice survived for 3 weeks after receiving the final dose. Mice also could not tolerate 2 weekly doses of 10 mg DMBA as only 2 male mice and 1 female mouse out of groups of 20 mice survived for 6 weeks after receiving the final dose of DMBA. Mice which received placebos survived for the term of the experiment.

The toxicity of DMBA was also reflected in the body weights of mice which received various amounts of DMBA in a

single dose (Figure 11). Gains in body weight were decreased in mice which had received greater amounts of DMBA. Survival times of mice were also decreased when mice received increased amounts of DMBA in a single dose (Table 10). Survival times of both male and female mice, with or without tumours at death, were negatively correlated with the amount of DMBA each mouse had received. These correlations were statistically significant. The mean survival times of mice with tumours were, in most cases, greater than those of mice of the same sex which had received the same amount of DMBA and were without tumours at death. These differences between the mean survival times were statistically significant (Student's t-test) in the following groups of animals: female mice, 15 mg DMBA, $P < 0.001$; male mice, 5 mg DMBA, $P < 0.05$. This result suggests that the mice which developed tumours were those which did not die earlier from the toxicity of the DMBA.

The number of mice which developed tumours after receiving various amounts of DMBA in a single dose and the total number of mice in each experimental group are shown in Table 11. The cumulative incidences of tumour-bearing male and female mice are shown in Figures 12 and 13 respectively. Increases in the amount of DMBA each mouse received were accompanied by increases in the cumulative incidence of tumour-bearing mice. This effect appeared more distinct in female mice than in male mice. As suggested by Peto (1974) the best test of the carcinogenicity of a substance given at various dose levels is to pool all the groups except the highest dose group. Only two groups are then compared: the highest dose group and the pooled lower dose and control groups. The difference between the cumulative incidence of

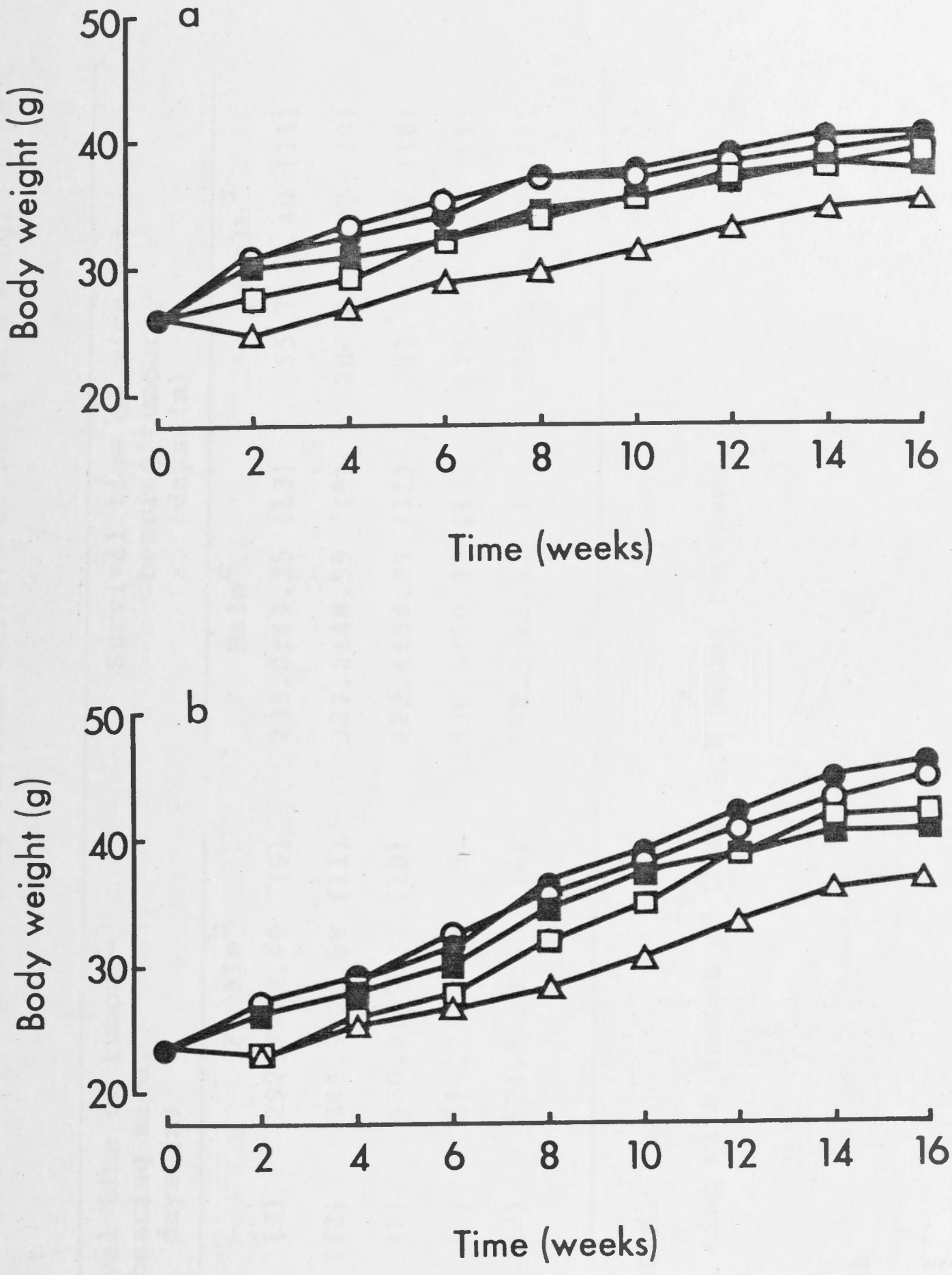


FIGURE 11. *Mean body weights of groups of 20 mice receiving various amounts of DMBA in a single dose.* a. Male mice. b. Female mice. Amount of DMBA: △—△, 15 mg; □—□, 5 mg; ■—■, 1.5 mg; ○—○, 0.5 mg; ●—●, none. Mice received a dose of DMBA or a placebo at week zero.

TABLE 10. Survival times of mice which received various amounts of DMBA in a single dose.

Amount of DMBA, mg	Survival time of tumour-bearing mice, days (n)		Survival time of mice not bearing tumours, days (n)	
	Male[a]	Female[b]	Male[c]	Female[d]
15.0	377.9±44.25 (7)	297.7±22.40 (9)	233.0±47.35 (13)	55.7±25.40 (11)
5.0	467.3±45.45 (11)	345.9±29.88 (11)	327.2±48.59 (9)	286.6±25.77 (9)
1.5	505.8±53.77 (9)	430.9±29.22 (10)	422.4±34.39 (11)	353.4±31.07 (10)
0.5	624.0±16.09 (7)	546.2±22.75 (9)	536.5±50.33 (13)	475.0±44.65 (11)
None	527.0±38.00 (2)	524.5±50.60 (6)	592.3±22.07 (18)	508.5±30.73 (14)

Each value is the mean±SEM.

Correlation of survival time with amount of DMBA each mouse received.

a $r = -0.4879$, $P < 0.01$

b $r = -0.6260$, $P < 0.001$

c $r = -0.6403$, $P < 0.001$

d $r = -0.8195$, $P < 0.001$

TABLE 11. Number of tumour-bearing mice among animals which received various amounts of DMBA in a single dose.

Sex	Amount of DMBA, mg	Time after DMBA administration, weeks												
		1-8	9-16	17-24	25-32	33-40	41-48	49-56	57-64	65-72	73-80	81-88	89-96	97-104
Female	15.0	0/20[a]	0/11	0/11	2/11	0/8	5/7	1/2	1/1	0/0	0/0	0/0	0/0	0/0[b]
	5.0	0/20	0/20	0/20	2/20	1/15	3/13	0/7	4/6	1/2	0/0	0/0	0/0	0/0
	1.5	0/20	0/20	0/20	0/20	1/18	2/16	0/13	2/12	1/7	4/4	0/0	0/0	0/0
	0.5	0/20	0/20	0/19	0/19	0/19	0/19	0/19	0/18	3/16	1/11	4/9	1/2	0/0
	None	0/20	0/20	0/20	0/20	0/20	1/20	0/18	0/16	1/16	3/13	0/4	0/4	1/2
Male	15.0	0/20	0/16	0/16	1/15	1/14	0/11	1/9	3/7	0/2	1/1	0/0	0/0	0/0
	5.0	0/20	0/20	1/20	0/17	0/17	1/15	1/14	1/12	2/10	2/5	1/3	2/2	0/0
	1.5	0/20	0/20	0/20	0/20	1/20	1/18	0/16	1/14	1/10	1/6	2/5	0/3	2/3
	0.5	0/20	0/20	0/20	0/19	0/19	0/19	0/18	0/17	0/16	1/15	1/13	4/9	1/4
	None	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	1/20	0/15	1/12	0/7	0/3

[a] x/y: x is the number of tumour-bearing mice which died during each 8 week period.
y is the number of mice alive at the beginning of each period.

[b] By week 104 all mice had died of natural causes.

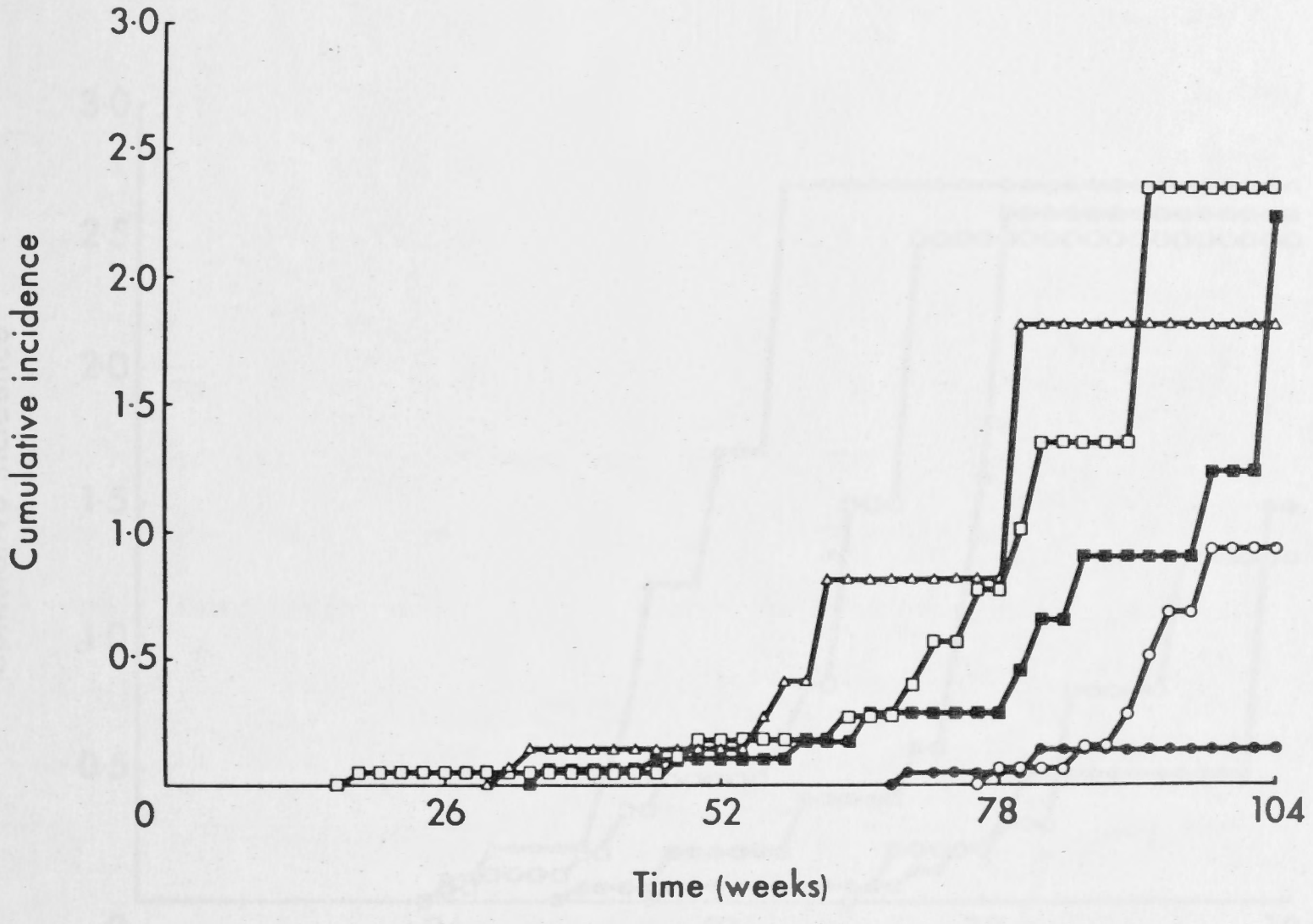


FIGURE 12. *Cumulative incidences of tumour-bearing mice among male mice receiving various amounts of DMBA in a single dose.* Amount of DMBA: △—△, 15 mg; □—□, 5 mg; ■—■, 1.5 mg; ○—○, 0.5 mg; ●—●, none.

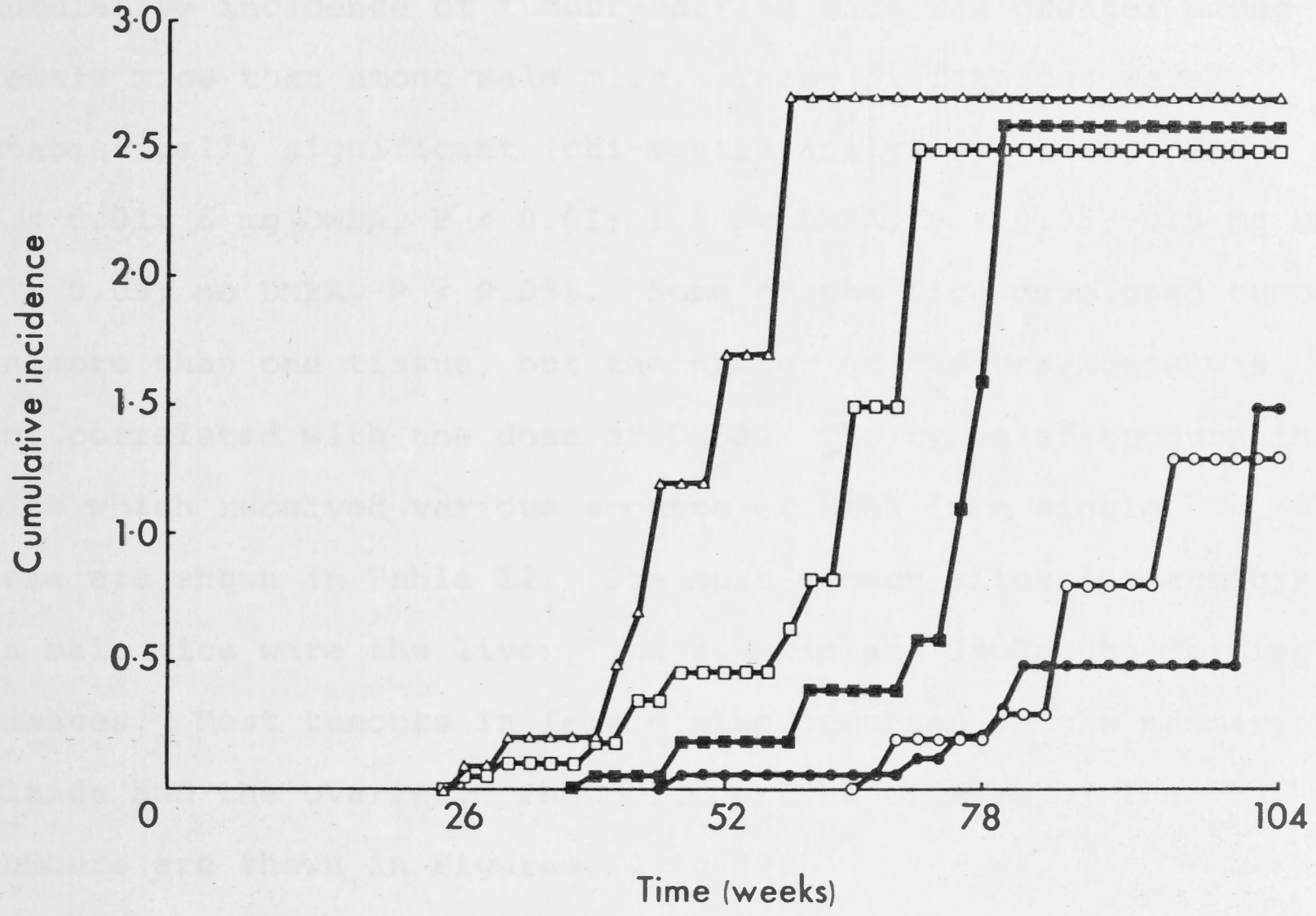


FIGURE 13. *Cumulative incidences of tumour-bearing mice among female mice receiving various amounts of DMBA in a single dose.* Amount of DMBA: △—△, 15 mg; □—□, 5 mg; ■—■, 1.5 mg; ○—○, 0.5 mg; ●—●, none.

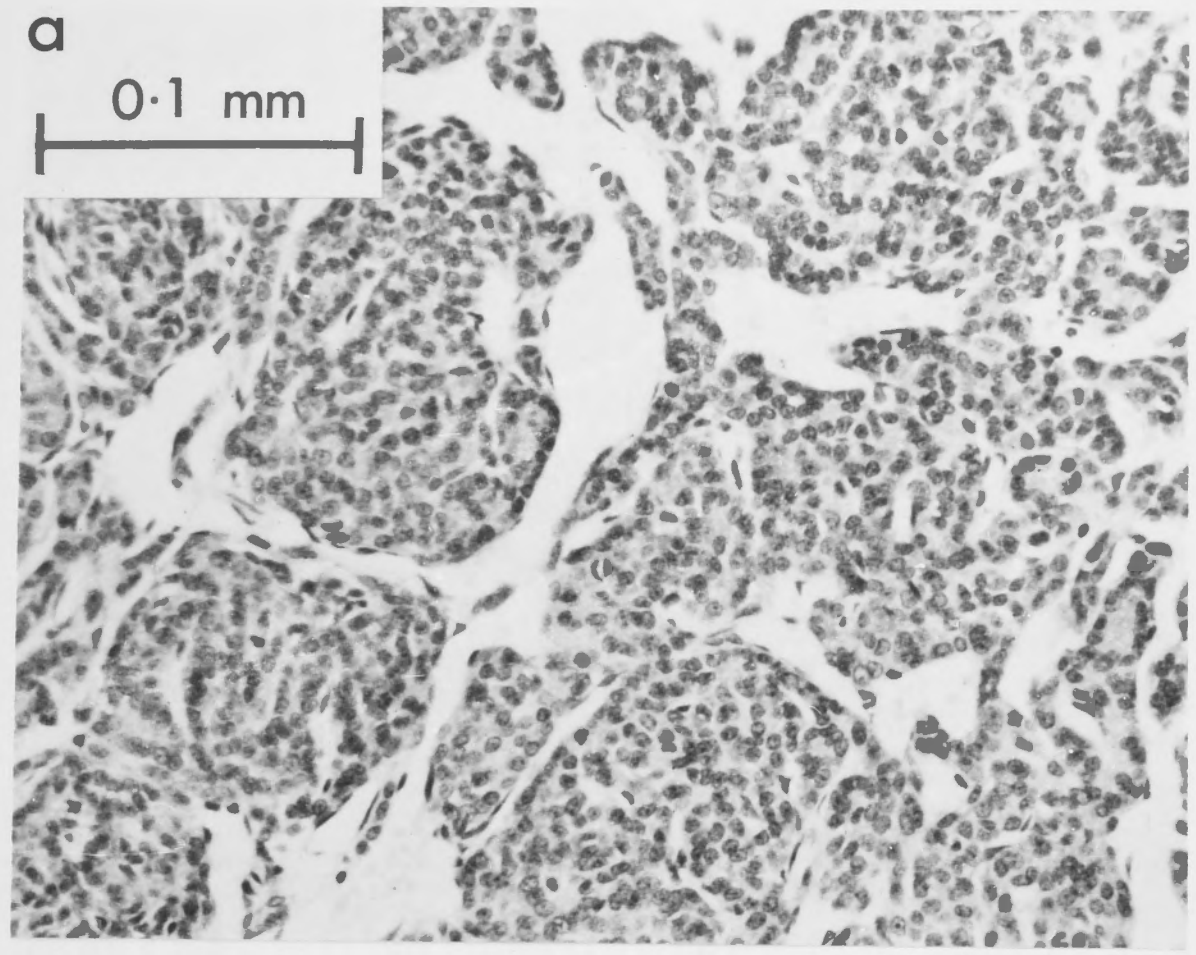
tumour-bearing mice among animals which received 15 mg DMBA and the pooled cumulative incidence of all other groups was statistically significant (chi-square analysis, $P < 0.001$) for both male and female mice. At each dose level of DMBA the cumulative incidence of tumour-bearing mice was greater among female mice than among male mice. These differences were statistically significant (chi-square analysis: 15 mg DMBA, $P < 0.01$; 5 mg DMBA, $P < 0.01$; 1.5 mg DMBA, $P < 0.05$; 0.5 mg DMBA, $P < 0.05$; no DMBA, $P < 0.05$). Some of the mice developed tumours in more than one tissue, but the number of tumours/mouse was not correlated with the dose of DMBA. The types of tumours in mice which received various amounts of DMBA in a single dose are shown in Table 12. The most common sites for tumours in male mice were the liver, lungs, skin and leukocyte-forming tissues. Most tumours in female mice occurred in the mammary glands and the ovaries. Photomicrographs of some of the tumours are shown in Figures 14 to 19.

The prime aim of this experiment was to determine a suitable dose of DMBA to induce tumours in mice fed the polyunsaturated and saturated fat diets. The number of mice which died of causes other than tumours was far greater in mice which received 15 mg DMBA, especially during the first 20 weeks of the experiment, than in mice which received smaller doses of DMBA (Table 11). Mice fed the polyunsaturated and saturated fat diets therefore received a single dose of 5 mg DMBA.

TABLE 12. Types of tumours in mice which received various amounts of DMBA in a single dose.

Tissue: type of tumour	Number of male mice with tumours — Amount of DMBA, mg					Number of female mice with tumours — Amount of DMBA, mg				
	15	5	1.5	0.5	None	15	5	1.5	0.5	None
Colon: papilloma				2						
Intestine: adenocarcinoma										1
Leukocyte-forming tissues:										
lymphoblastic leukaemia	1	2	1	2					1	1
lymphocytic leukaemia	1	1		1	1			1		2
myeloid leukaemia				1						
Liver: liver cell adenoma			1							
hepatocellular carcinoma		1		1	1	1				
haemangioma	2	1								
Lung: adenocarcinoma		1	2							1
adenoma		3	4	1					1	1
Mammary gland: adenoacanthoma							1			
adenocarcinoma type A						3		2		
adenocarcinoma type B							2	2	2	
Ovary: granulosa cell tumour						3	7	2	3	
mixed granulosa-thecal cell tumour						2			1	
reticulum cell sarcoma									1	
spindle cell tumour								2	1	
Skin: basal cell carcinoma							1			
squamous cell carcinoma	2	2	3							
baso-squamous cell carcinoma			1					1		
Stomach: squamous cell carcinoma	1	1						1		
Subcutaneous tissue: fibrosarcoma			1							
Uterus: fibroma							1			

Some mice developed more than one tumour each.



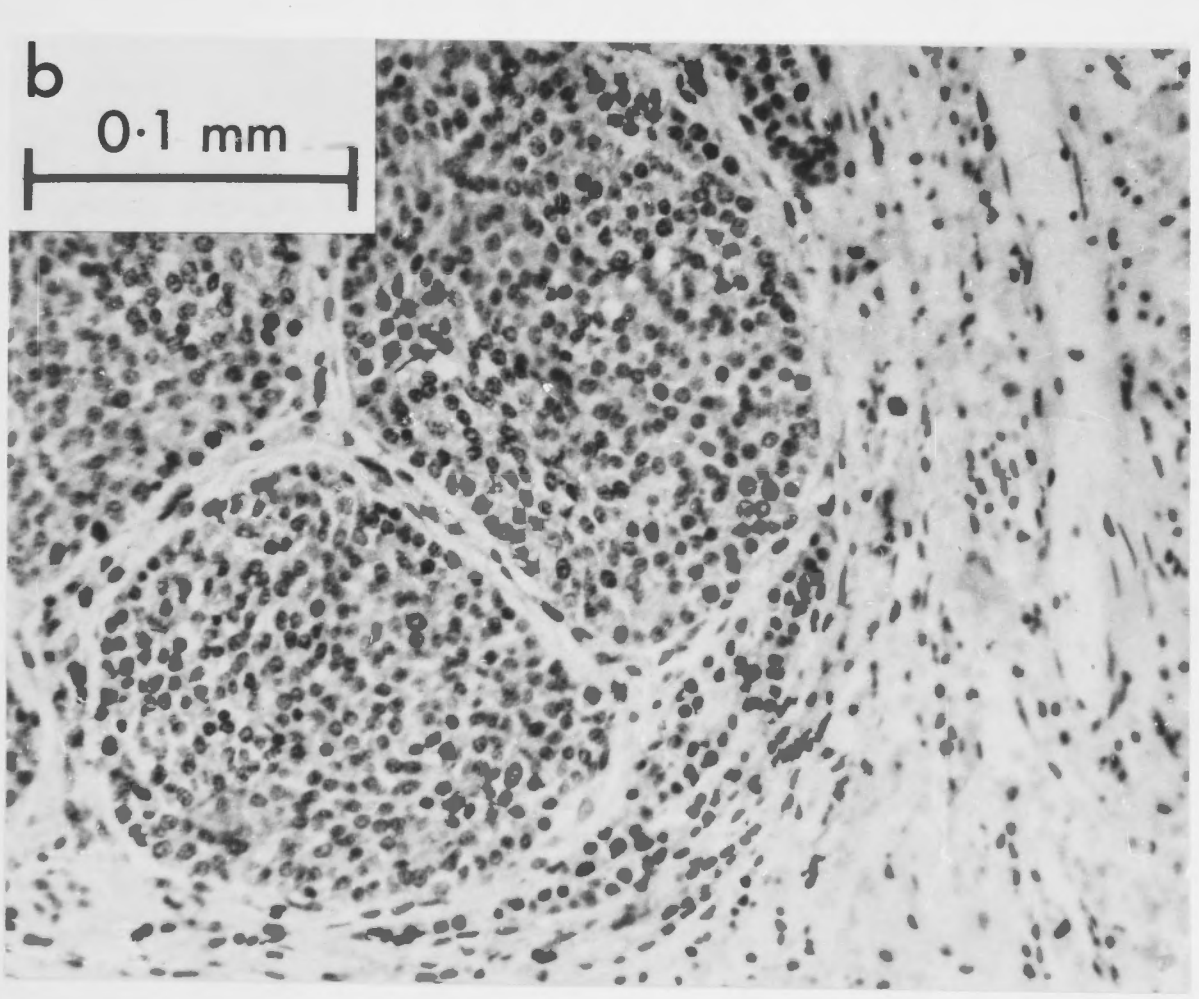
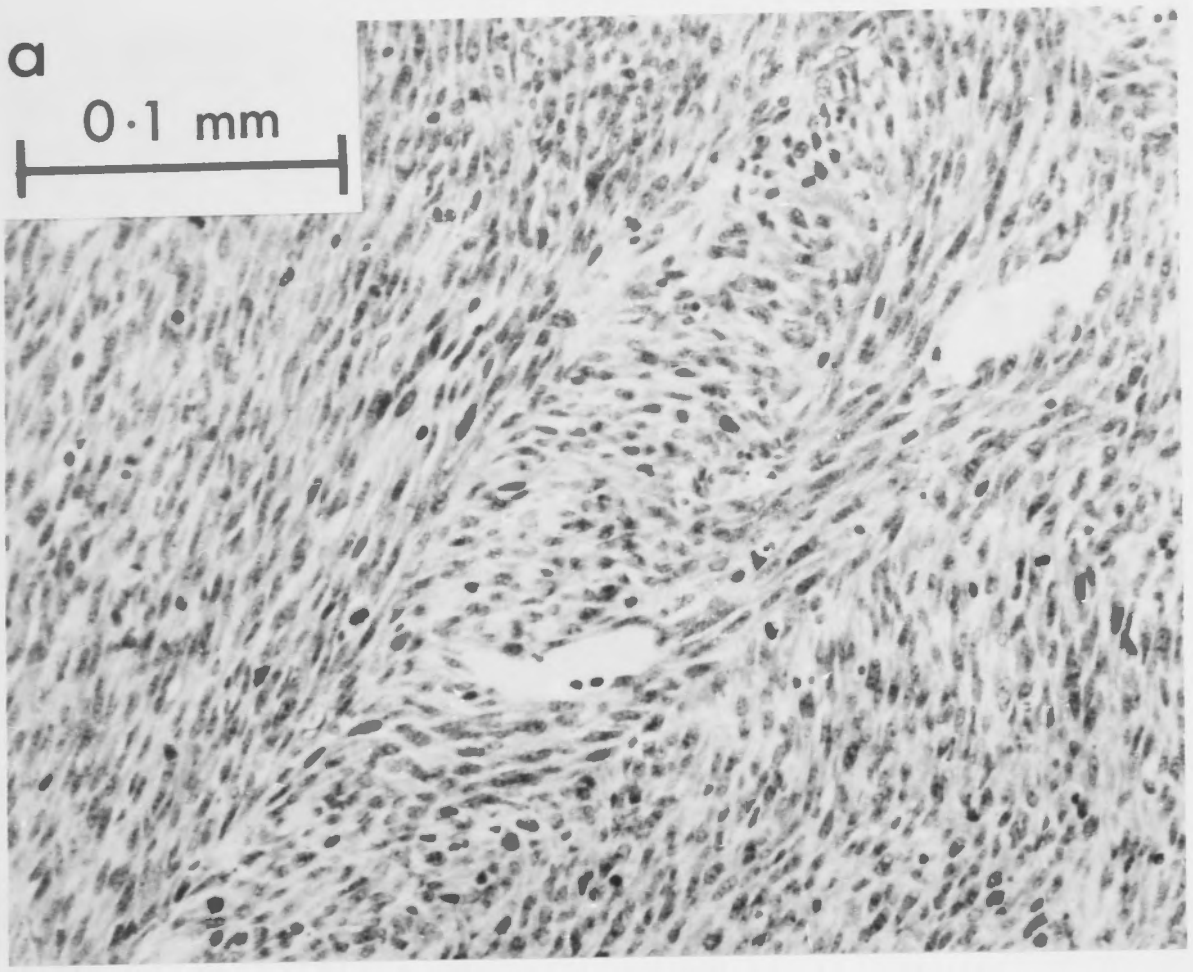


FIGURE 14. *Photomicrographs of mouse tumours.* a. *Granulosa cell tumour of ovary.* Haematoxylin and eosin; magnification 330X. b. *Metastases of ovarian granulosa cell tumour in lung.* Haematoxylin and eosin; magnification 330X. Figures 14 to 19 by courtesy of Dr G.C. Hard.



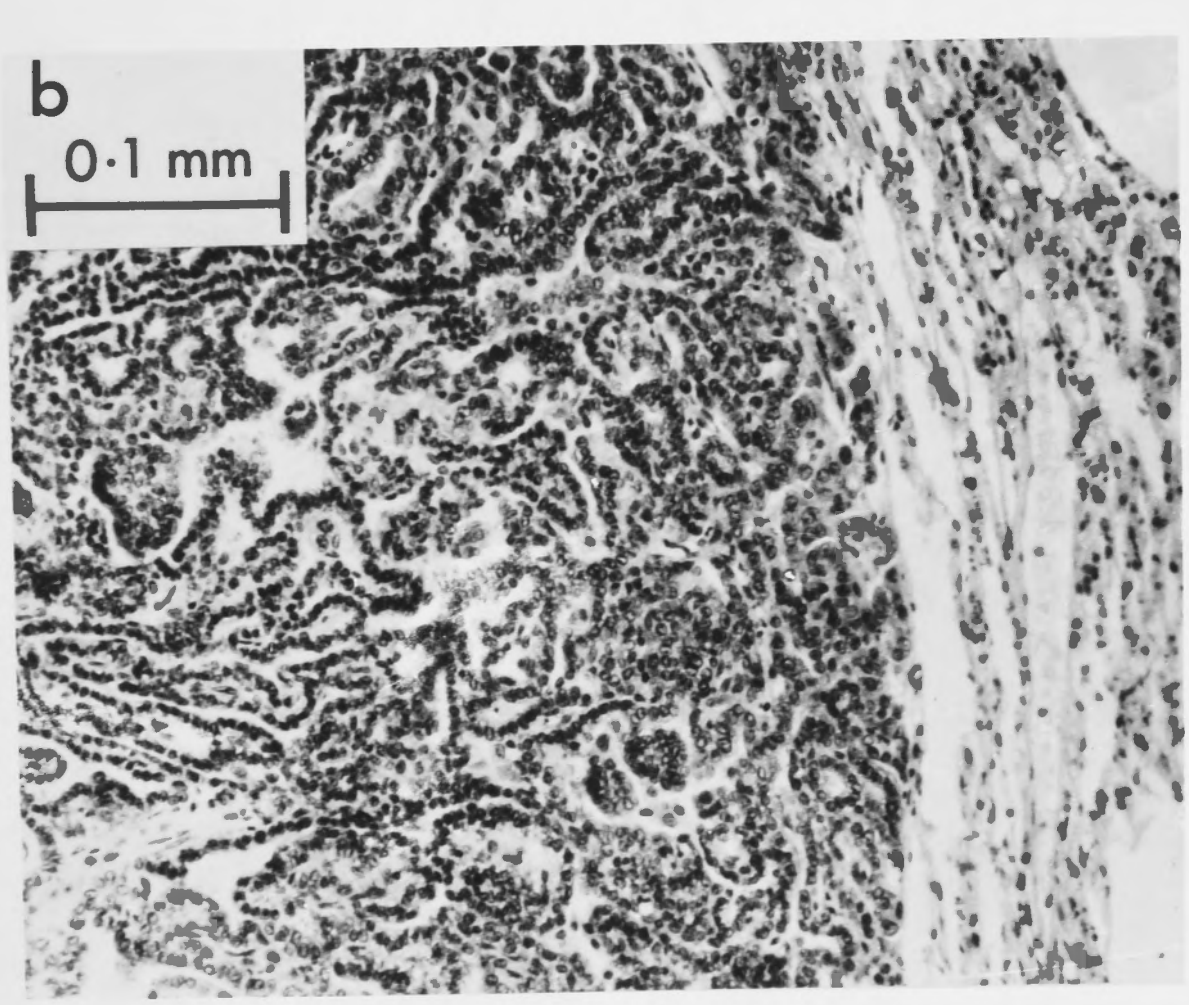
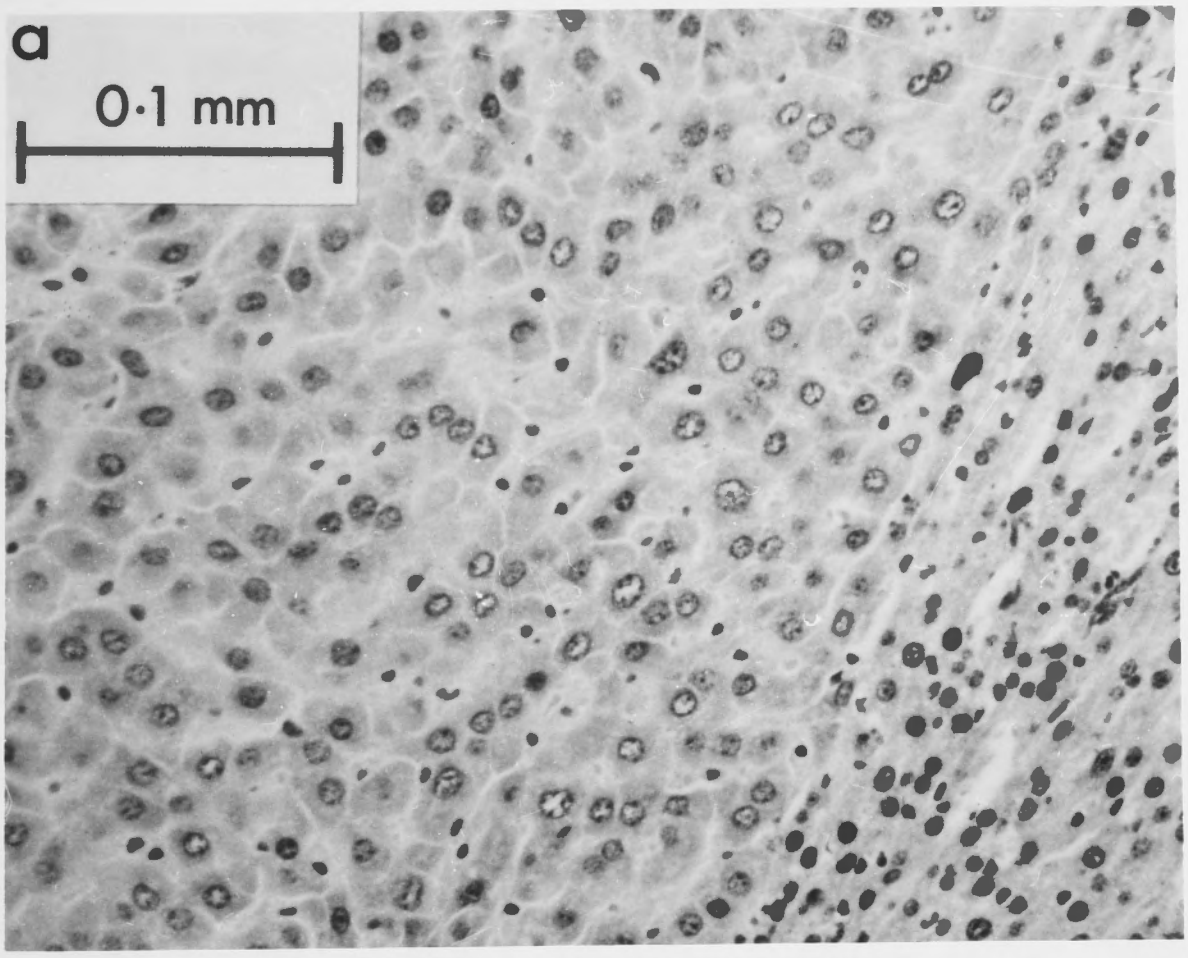


FIGURE 15. *Photomicrographs of mouse tumours.* a. *Fibrosarcoma of subcutaneous tissue.* Haematoxylin and eosin; magnification 330X. b. *Pulmonary adenoma.* Note the normal lung tissue to the right. Haematoxylin and eosin; magnification 260X.



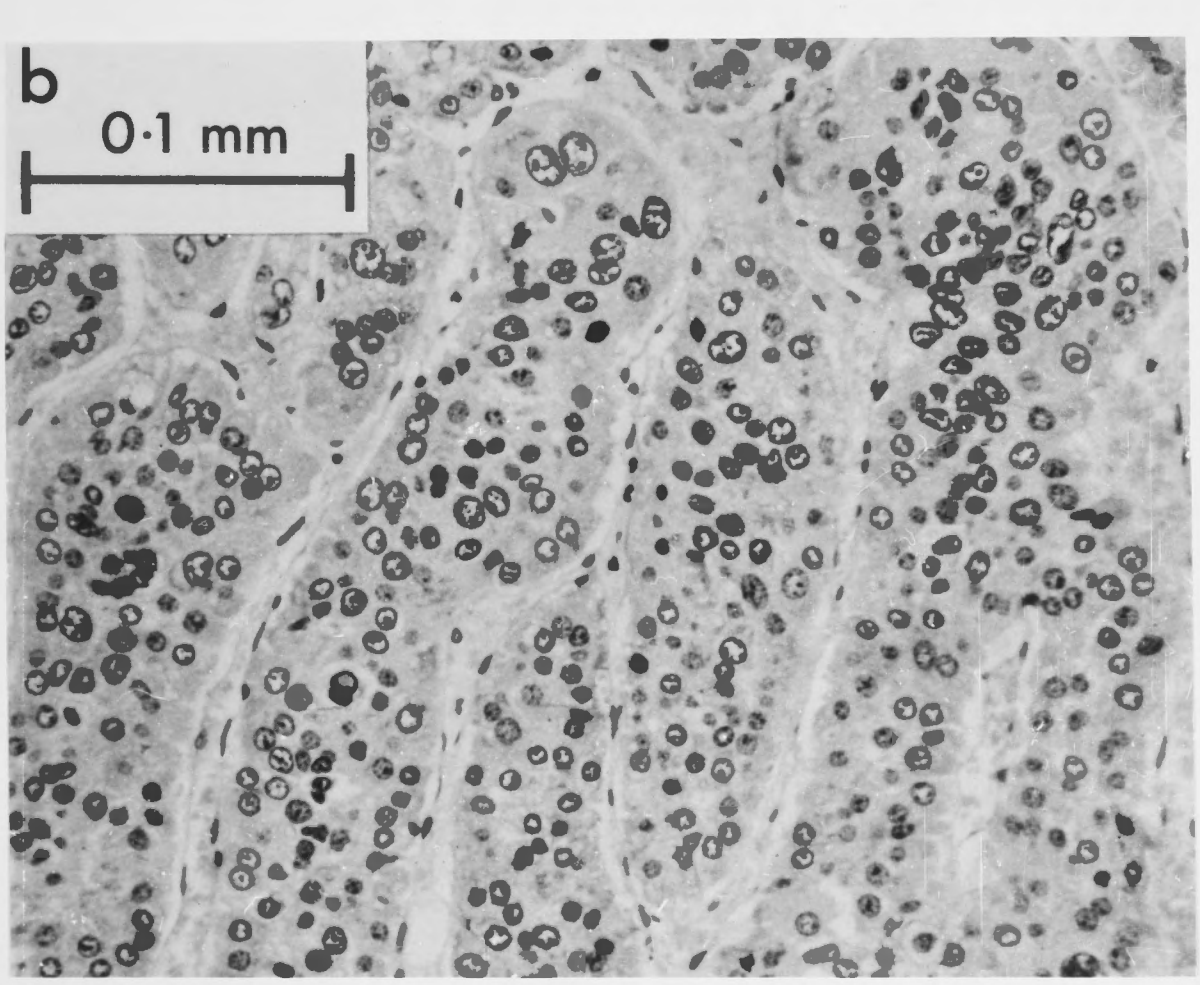
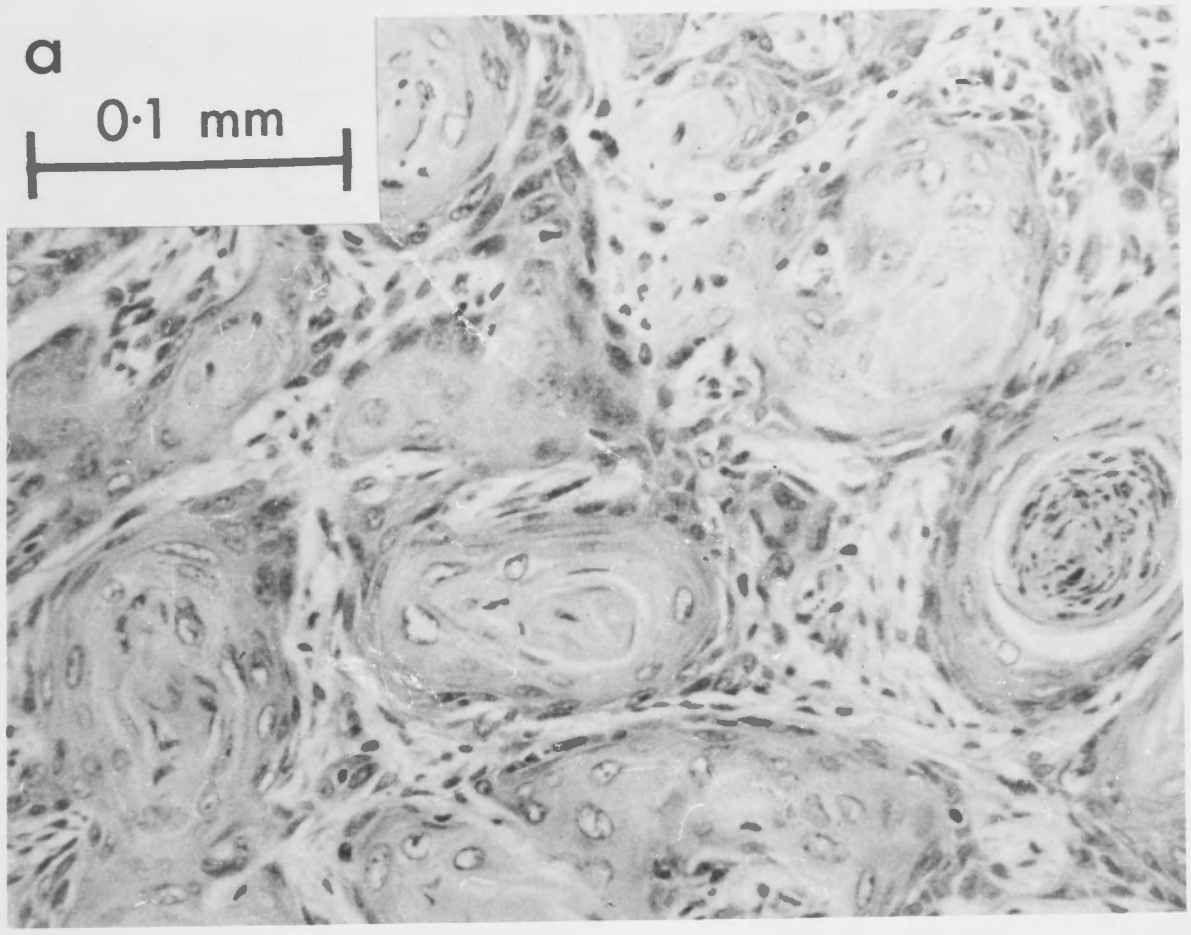


FIGURE 16. *Photomicrographs of mouse tumours.* a. *Liver cell adenoma.* Note the compressed normal tissue to the right. Haematoxylin and eosin; magnification 330X. b. *Hepatocellular carcinoma.* Haematoxylin and eosin; magnification 330X.



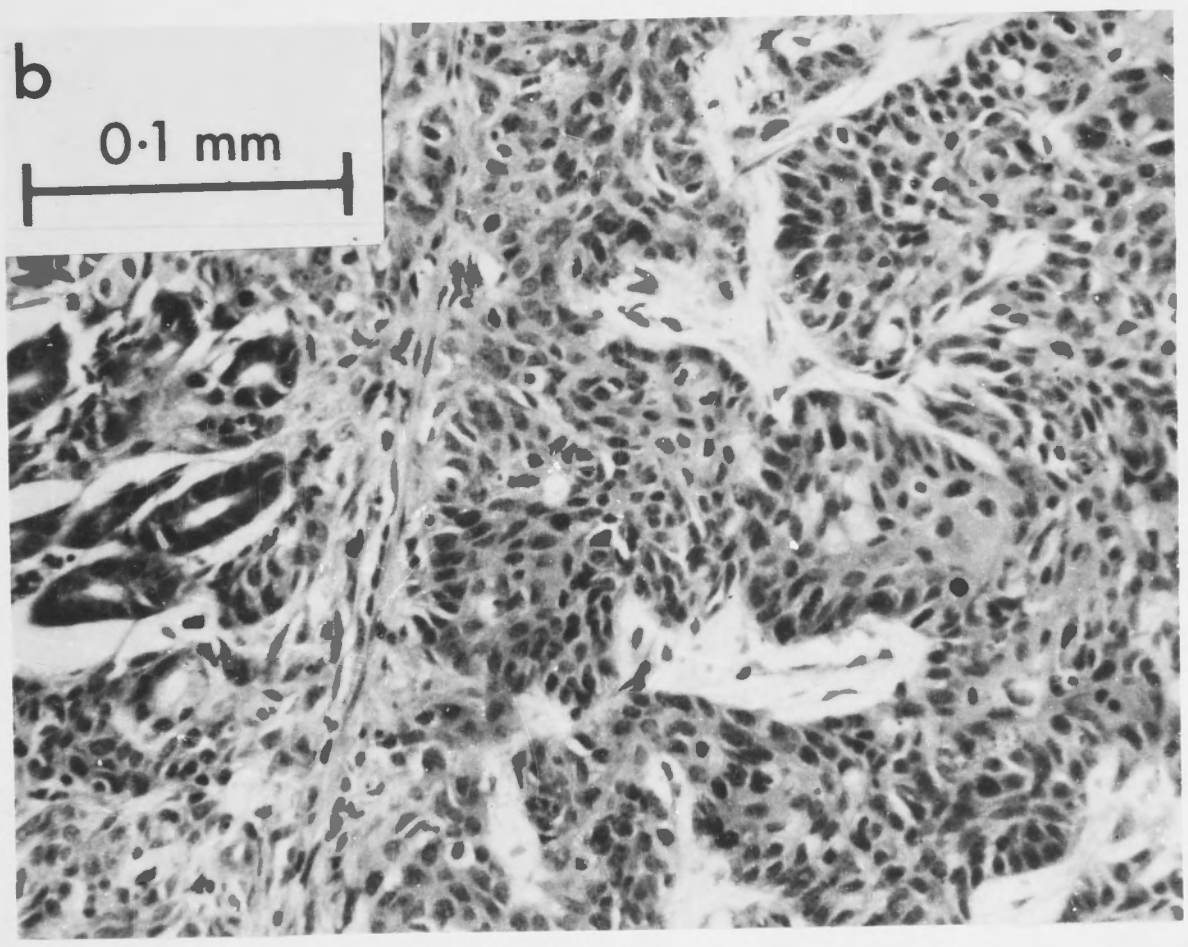
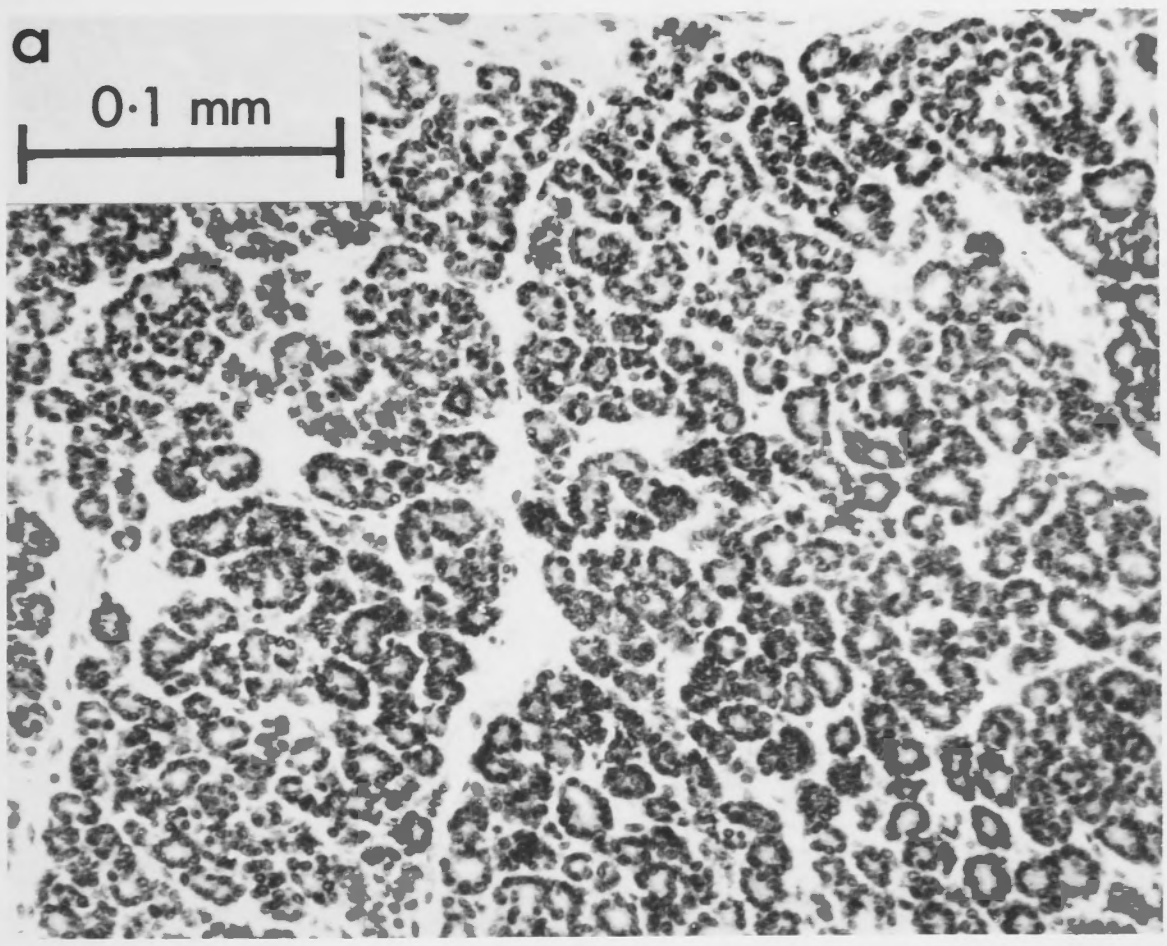


FIGURE 17. *Photomicrographs of mouse tumours.* a. *Squamous cell carcinoma of skin.* Haematoxylin and eosin; magnification 330X. b. *Squamous cell carcinoma of stomach.* Note the normal glandular lining at the left. Haematoxylin and eosin; magnification 330X.



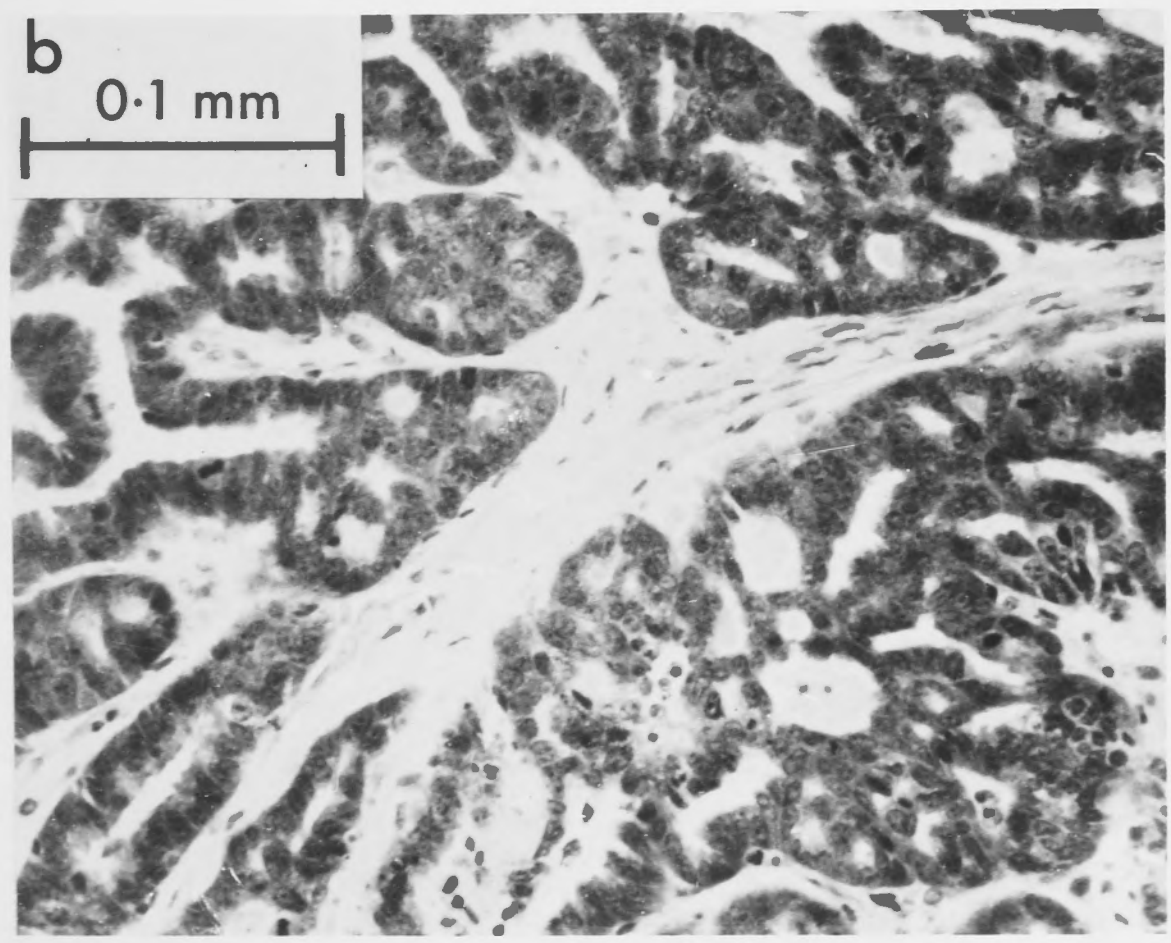
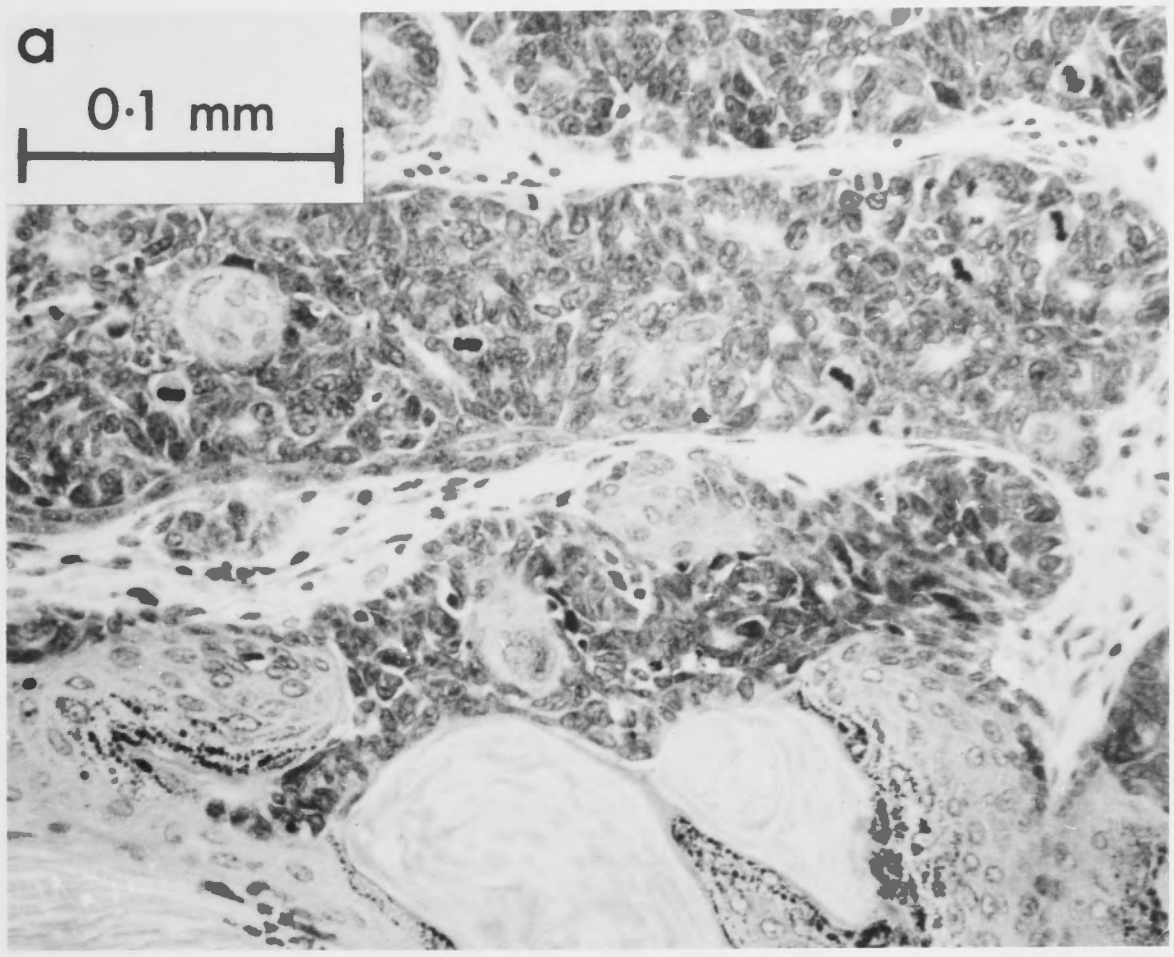


FIGURE 18. *Photomicrographs of mouse tumours.* a. *Adenocarcinoma type A of mammary gland.* Note the characteristic acinar structure. Haematoxylin and eosin; magnification 330X. b. *Adenocarcinoma type B of mammary gland.* Note the characteristic papillary, epithelial structure. Haematoxylin and eosin; magnification 330X.



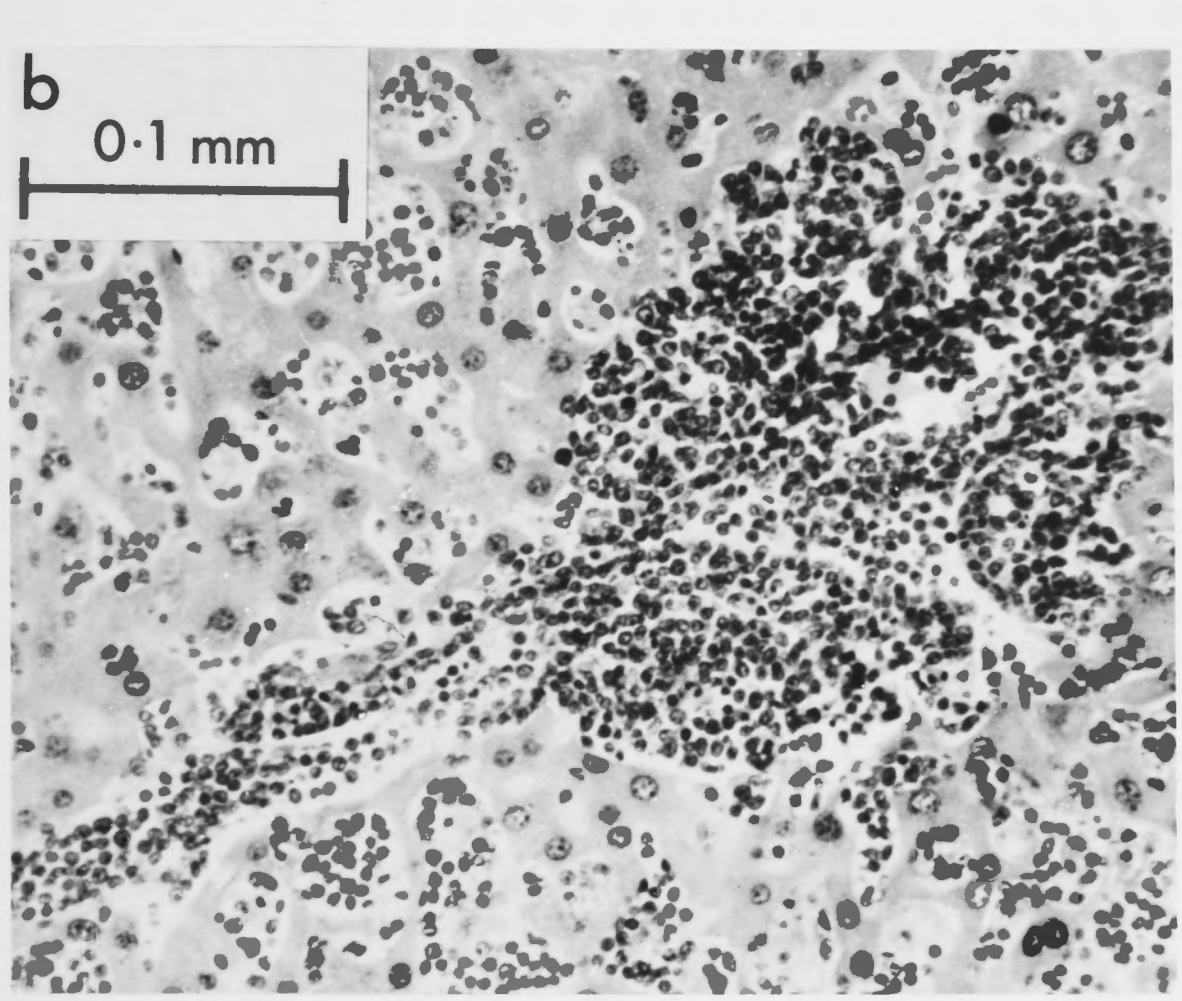


FIGURE 19. *Photomicrographs of mouse tumours.* a. *Adeno-acanthoma of mammary gland.* Note characteristic mixture of glandular tumour tissue and squamous epithelium with keratinisation. Haematoxylin and eosin; magnification 330X. b. *Lymphocytic leukaemia - infiltration of liver.* Haema-toxylin and eosin; magnification 330X.

(ii) Tumours in mice receiving different dietary fats

There were no significant differences in growth rate, as assessed by body weights, between mice of the same sex fed either the polyunsaturated or the saturated fat diet (Figure 20). These mice were not used in the study of the effect of these diets on DMBA-induced carcinogenesis. Some of the DMBA-dosed and control mice used in this study were weighed weekly for the first 20 weeks of the experiment. As with the DMBA-dosed rats (Section 2), there were no differences in body weights between mice of the same sex fed either the polyunsaturated or the saturated fat diet. When the mice were fed a different diet after DMBA administration the ensuing growth rate was not altered.

The number of mice which developed tumours and the total number of mice in each experimental group are shown in Table 13. The final cumulative incidence of tumour-bearing mice among DMBA-dosed female mice fed the saturated fat diet before and the polyunsaturated fat diet after DMBA administration was 2.4 fold that among DMBA-dosed female mice fed the saturated fat diet throughout the experiment (Figure 21). This difference was statistically significant (chi-square analysis, $P < 0.01$). Conversely, the final cumulative incidence of tumour-bearing mice among DMBA-dosed female mice fed the polyunsaturated fat diet throughout the experiment was 2.1 fold that among DMBA-dosed female mice switched to the saturated fat diet after DMBA administration (Figure 22). This difference was also statistically significant (chi-square analysis, $P < 0.05$). The final cumulative incidence of tumour-bearing mice among DMBA-dosed female mice fed the polyunsaturated fat diet throughout the experiment was 1.9 fold that among those fed the saturated fat diet throughout the experiment (Figure 23).

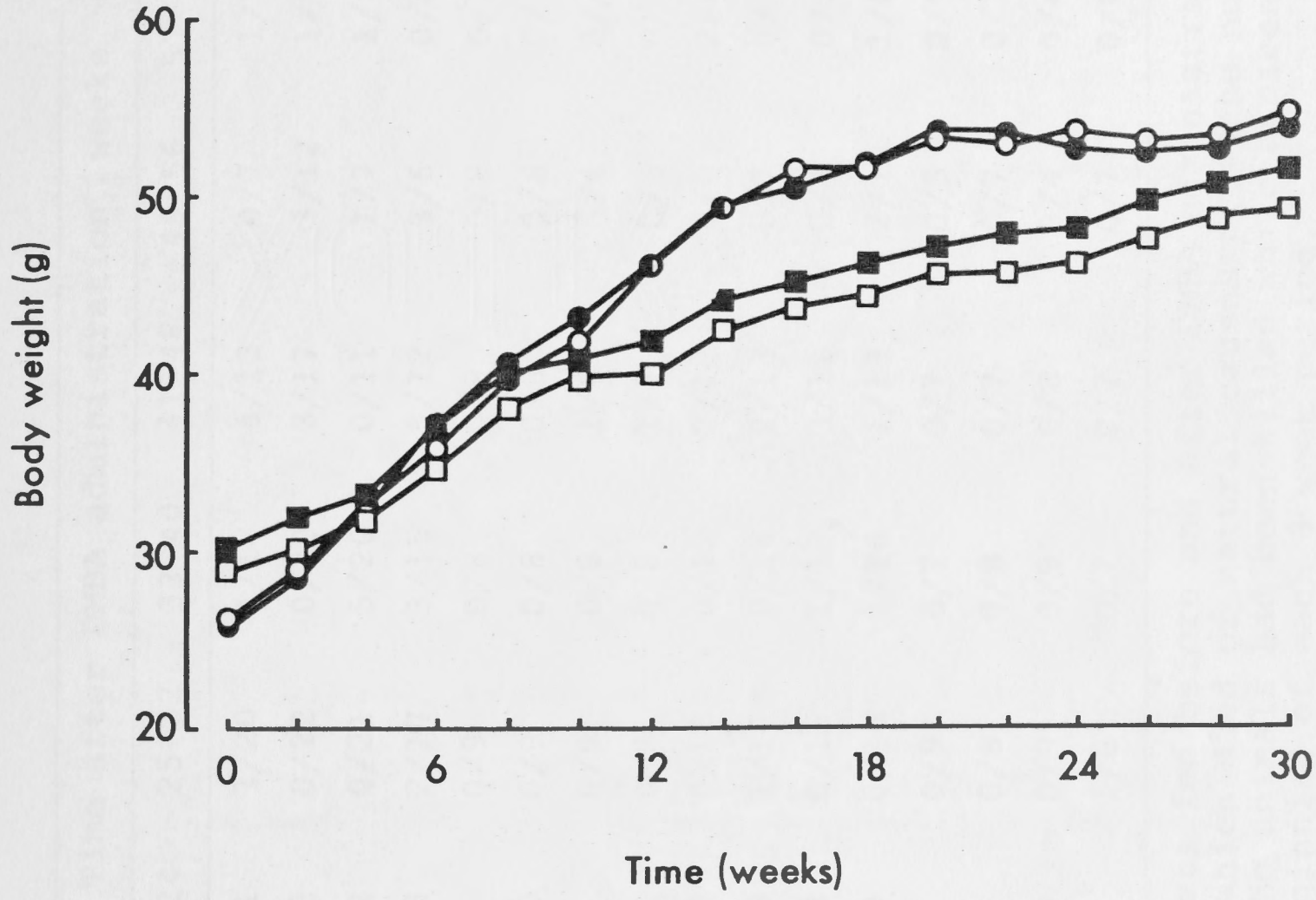


FIGURE 20. *Mean body weights of male mice fed the polyunsaturated fat diet (■—■) or the saturated fat diet (□—□), and female mice fed the polyunsaturated fat diet (●—●) or the saturated fat diet (○—○).* Each value is the mean of determinations on 35 to 38 mice.

TABLE 13. Number of tumour-bearing mice among animals fed the polyunsaturated fat diet (P) or the saturated fat diet (S).

Sex	Dose of DMBA, mg	Diet	Time after DMBA administration, weeks									
			1-8	9-16	17-24	25-32	33-40	41-48	49-56	57-64	65-72	73-80
Female	5	PP[a]	0/27[b]	2/26	0/21	3/20	4/17	4/13	0/7	1/7	1/5	2/4[c]
		SS	0/27	1/27	1/25	0/22	0/20	3/17	3/12	1/8	1/6	0/3
		PS	0/25	0/25	0/22	0/21	5/20	0/11	1/7	1/6	1/5	0/2
		SP	0/22	1/22	0/18	2/17	3/15	5/12	5/6	0/0	0/0	0/0
	None	PP	0/10	1/10	0/9	0/9	0/8	0/8	0/8	0/8	1/7	0/3
		SS	0/10	0/10	0/10	0/9	0/8	0/8	0/8	0/8	2/8	1/3
		PS	0/9	0/9	0/9	0/9	0/8	1/6	0/4	0/4	0/4	0/2
		SP	0/10	0/10	0/9	0/8	0/8	1/7	0/6	0/2	0/2	0/1
Male	5	PP	0/17	0/17	0/14	0/13	0/12	2/12	0/8	2/6	2/4	0/1
		SS	0/20	0/19	0/18	1/17	0/14	2/13	0/7	0/7	0/6	1/6
		PS	0/23	0/22	0/18	0/16	1/13	3/11	0/4	0/3	1/3	0/1
		SP	0/21	0/20	1/19	0/16	1/16	1/13	2/10	1/8	3/5	2/2
	None	PP	0/9	0/9	0/9	0/9	0/7	0/7	0/5	0/5	0/5	0/3
		SS	0/10	0/9	0/8	0/8	0/8	0/7	0/7	0/7	0/6	1/6
		PS	0/10	0/10	0/10	0/9	0/9	0/8	0/4	0/4	0/4	1/4
		SP	0/9	0/9	0/9	1/8	0/7	0/7	0/7	0/6	0/6	0/5

[a] The first and second letters refer to the diets fed before and after DMBA administration, respectively.
[b] x/y: x is the number of tumour-bearing mice which died of natural causes plus the number of mice with tumours discovered at autopsy after the animals had been killed while apparently healthy.
y is the number of mice alive at the beginning of each 8 week period.
[c] The mice did not develop tumours after week 80.

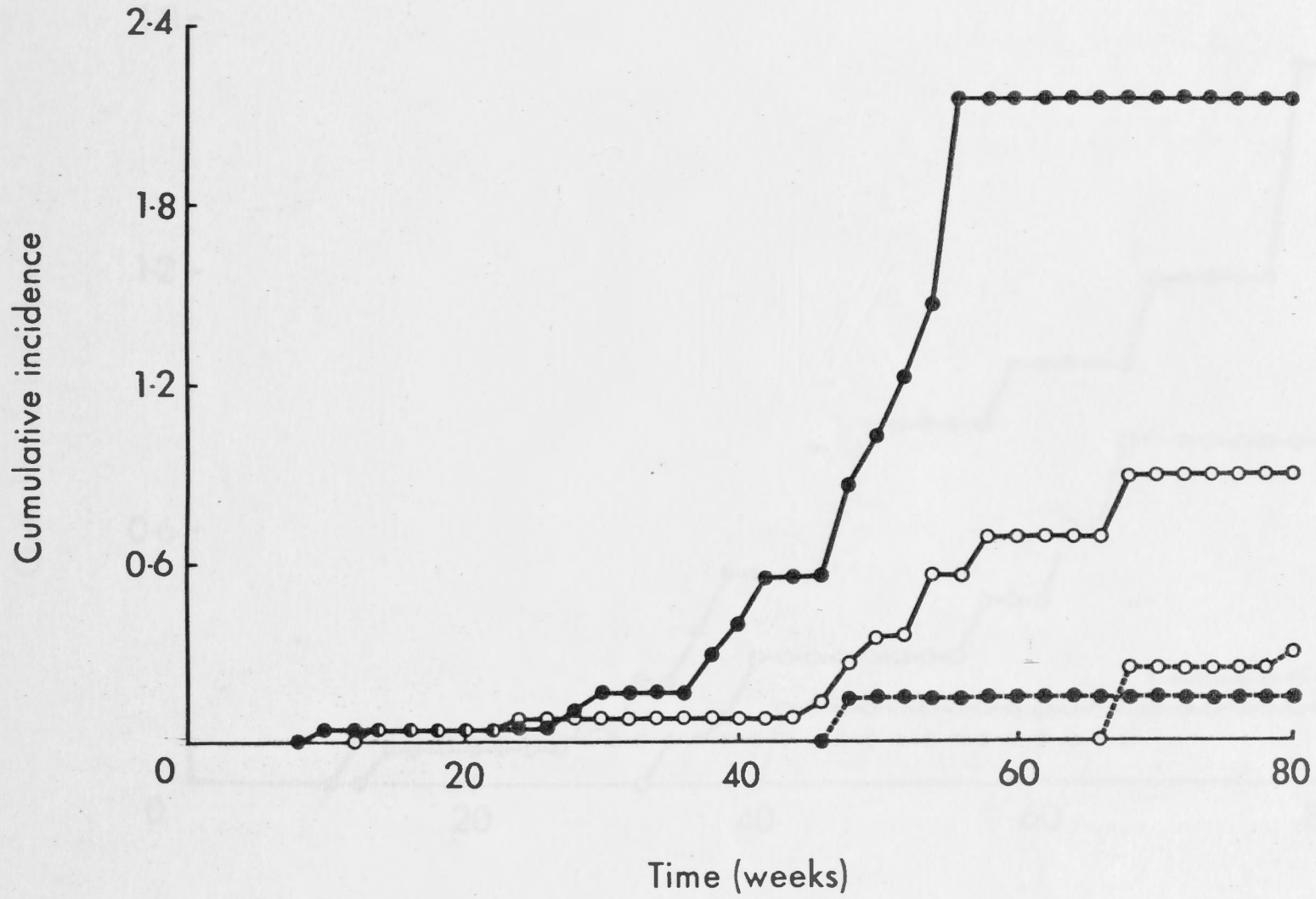


FIGURE 21. *Cumulative incidences of tumour-bearing female mice among DMBA-dosed (●—●) and control (○—○) mice fed the saturated fat diet throughout the experiment, and DMBA-dosed (●—●) and control (●—●) mice fed the saturated fat diet before and the polyunsaturated fat diet after DMBA administration.* The difference between the cumulative incidences among DMBA-dosed mice was statistically significant (chi-square analysis : $P < 0.01$).

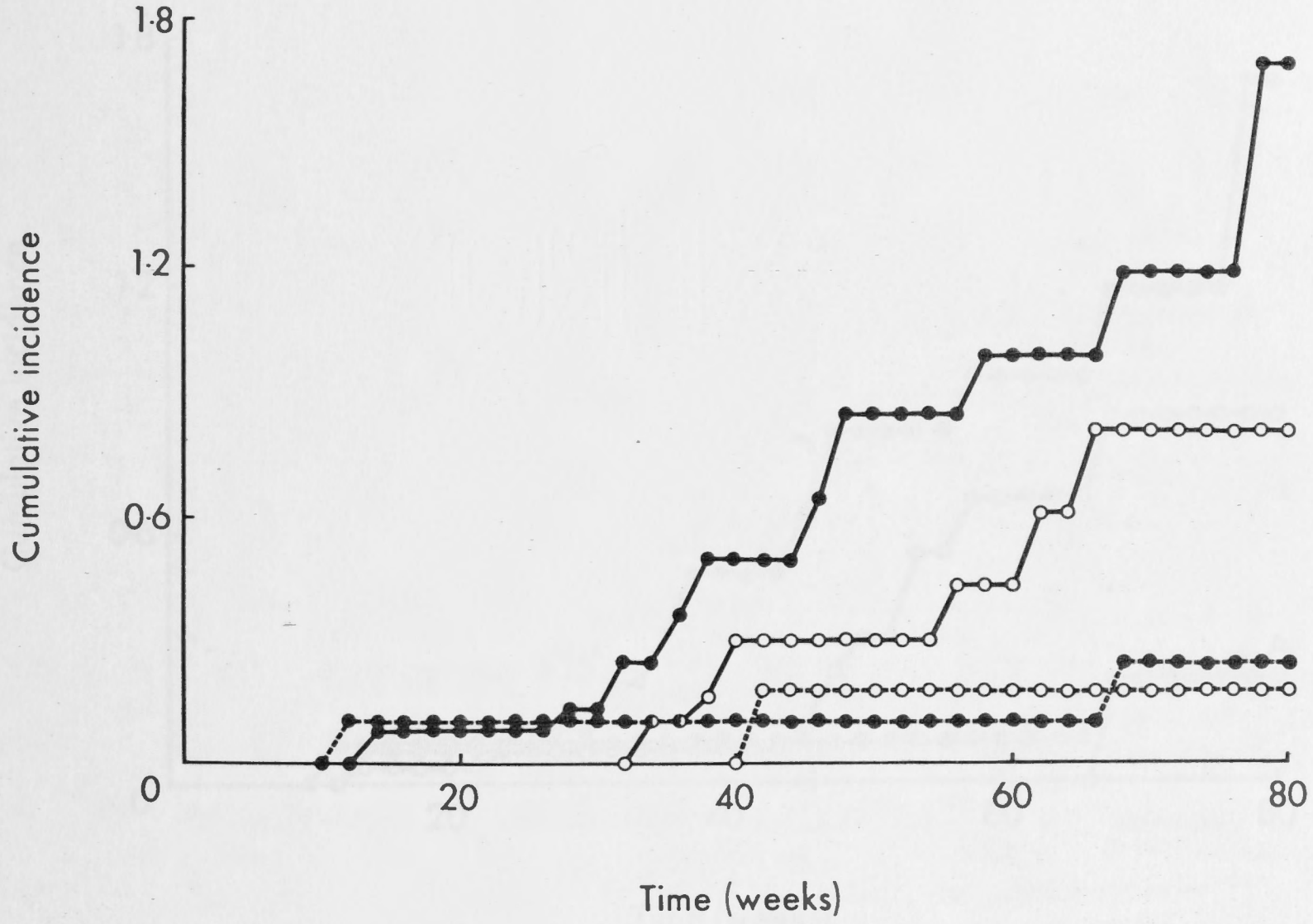


FIGURE 22. *Cumulative incidences of tumour-bearing female mice among DMBA-dosed (●—●) and control (●- -●) mice fed the polyunsaturated fat diet throughout tne experiment, and DMBA-dosed (○—○) and control (○- -○) mice fed the polyunsaturated fat diet before and the saturated fat diet after DMBA administration.* The difference between the cumulative incidences among DMBA-dosed mice was statistically significant (chi-square analysis : $P < 0.05$).

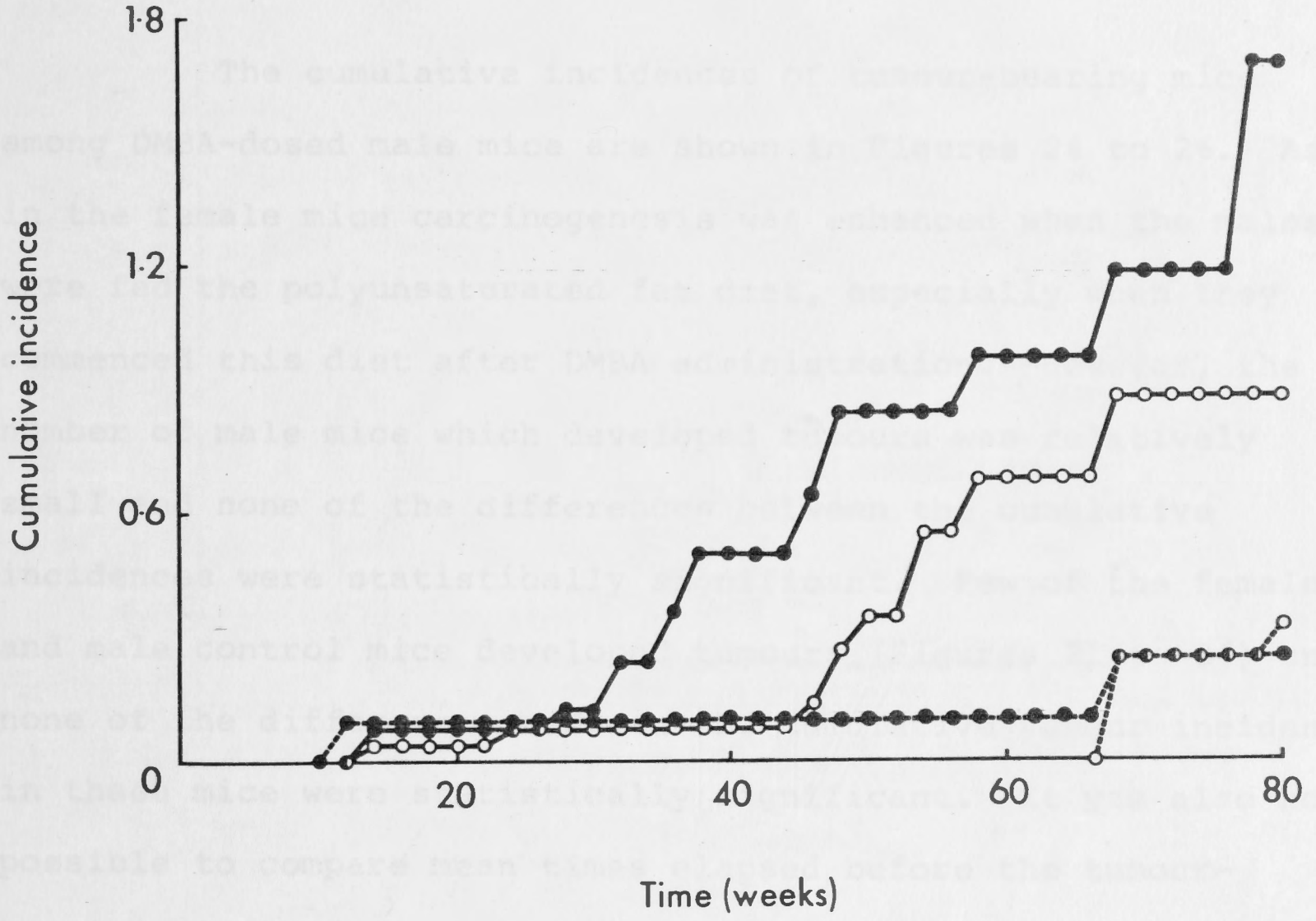


FIGURE 23. *Cumulative incidences of tumour-bearing female mice among DMBA-dosed (●—●) and control (●- -●) mice fed the polyunsaturated fat diet throughout the experiment, and DMBA-dosed (○—○) and control (○- -○) mice fed the saturated fat diet throughout the experiment.* The difference between the cumulative incidences among DMBA-dosed mice was statistically significant (chi-square analysis : $P < 0.05$).

This difference was statistically significant (chi-square analysis, $P < 0.05$). There were no statistically significant differences between the tumour incidences in mice fed different diets before DMBA administration and the same diet after DMBA administration.

The cumulative incidences of tumour-bearing mice among DMBA-dosed male mice are shown in Figures 24 to 26. As in the female mice carcinogenesis was enhanced when the males were fed the polyunsaturated fat diet, especially when they commenced this diet after DMBA administration. However, the number of male mice which developed tumours was relatively small and none of the differences between the cumulative incidences were statistically significant. Few of the female and male control mice developed tumours (Figures 21 to 26) and none of the differences between the cumulative tumour incidences in these mice were statistically significant. It was also not possible to compare mean times elapsed before the tumour-bearing mice had died as some of the mice had been killed while apparently healthy. Some of the mice also developed more than one tumour each, but there were no differences in the number of tumours/mouse when mice were fed the different diets. Tumours were not classified as malignant or benign as pathologists have found difficulty in reaching a consensus on the criteria for the classification of malignant and benign tumours in some tissues of the mouse (G.C. Hard, Baker Medical Research Institute, Prahran Vic 3181, personal communication). It was therefore not possible to compare the numbers of tumour-bearing mice which developed malignant tumours when mice were fed the different diets.

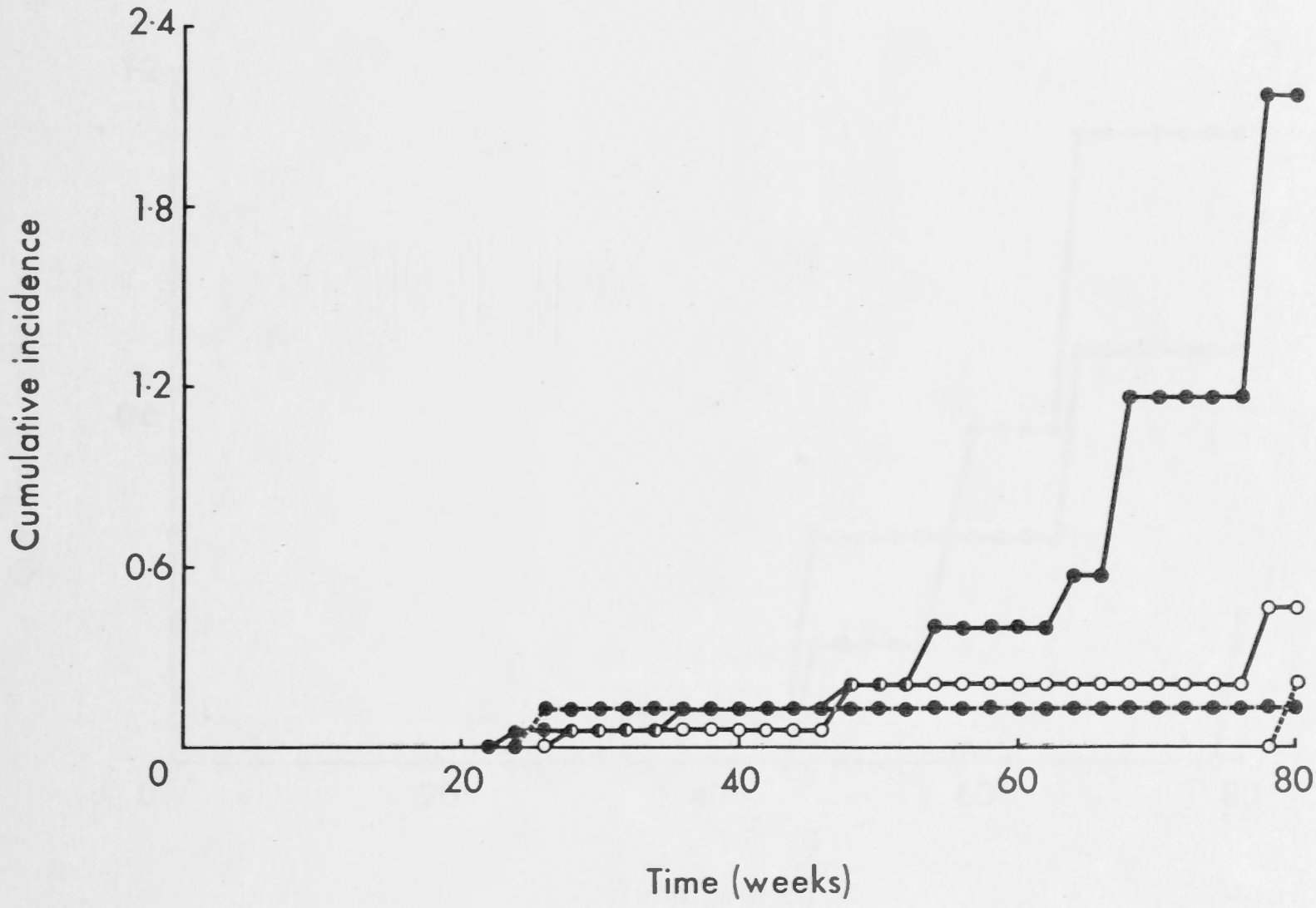


FIGURE 24. *Cumulative incidences of tumour-bearing male mice among DMBA-dosed (●—●) and control (○—○) mice fed the saturated fat diet throughout the experiment, and DMBA-dosed (●—●) and control (●—●) mice fed the saturated fat diet before and the polyunsaturated fat diet after DMBA administration.* The differences between the cumulative incidences among DMBA-dosed mice and among control mice were not statistically significant.

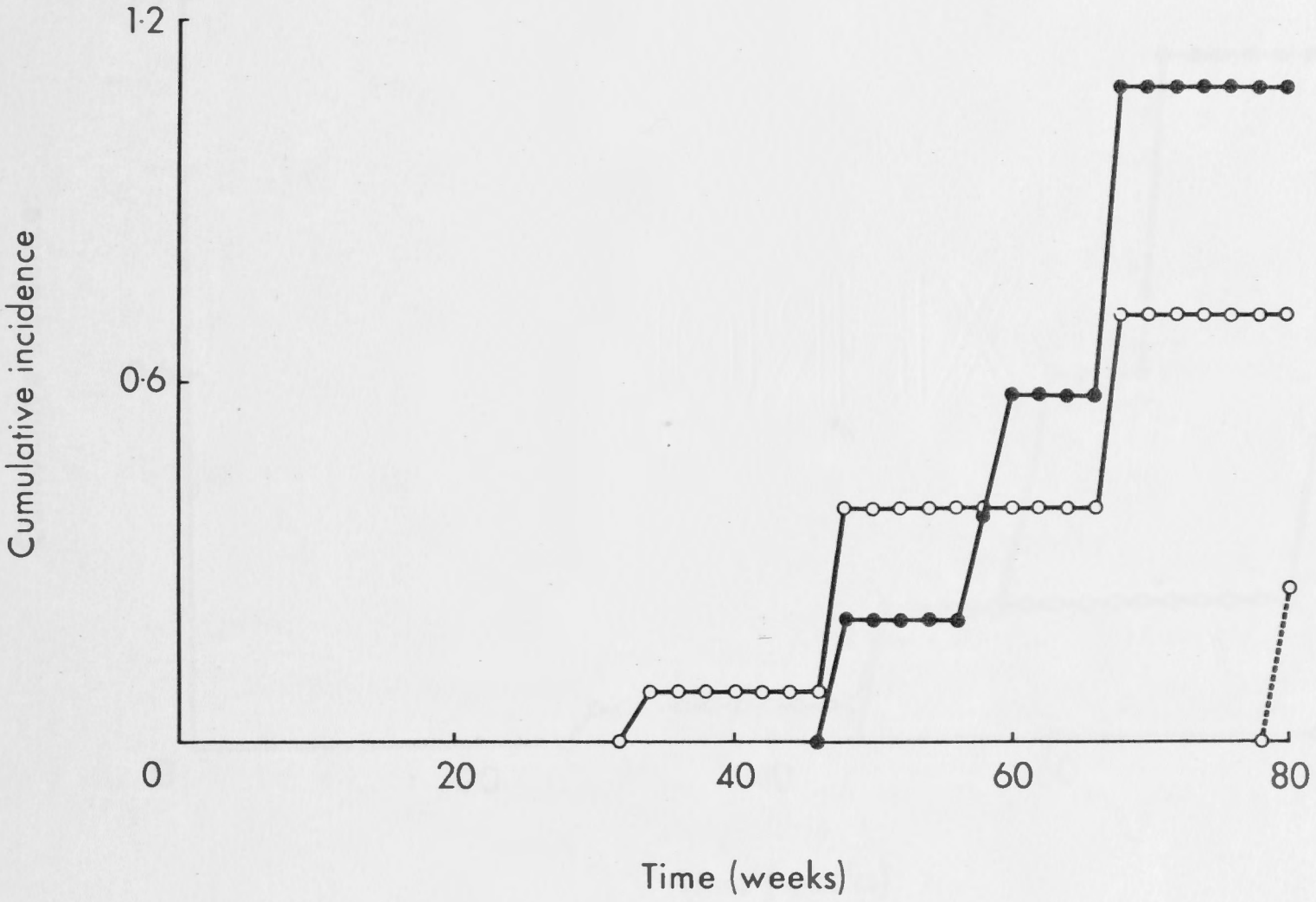


FIGURE 25. *Cumulative incidences of tumour-bearing male mice among DMBA-dosed mice fed the polyunsaturated fat diet throughout the experiment (●—●), and DMBA-dosed (○—○) and control (○– –○) mice fed the polyunsaturated fat diet before and the saturated fat diet after DMBA administration.* None of the control mice fed the polyunsaturated fat diet throughout the experiment developed tumours. The difference between the cumulative incidences among DMBA-dosed mice was not statistically significant.

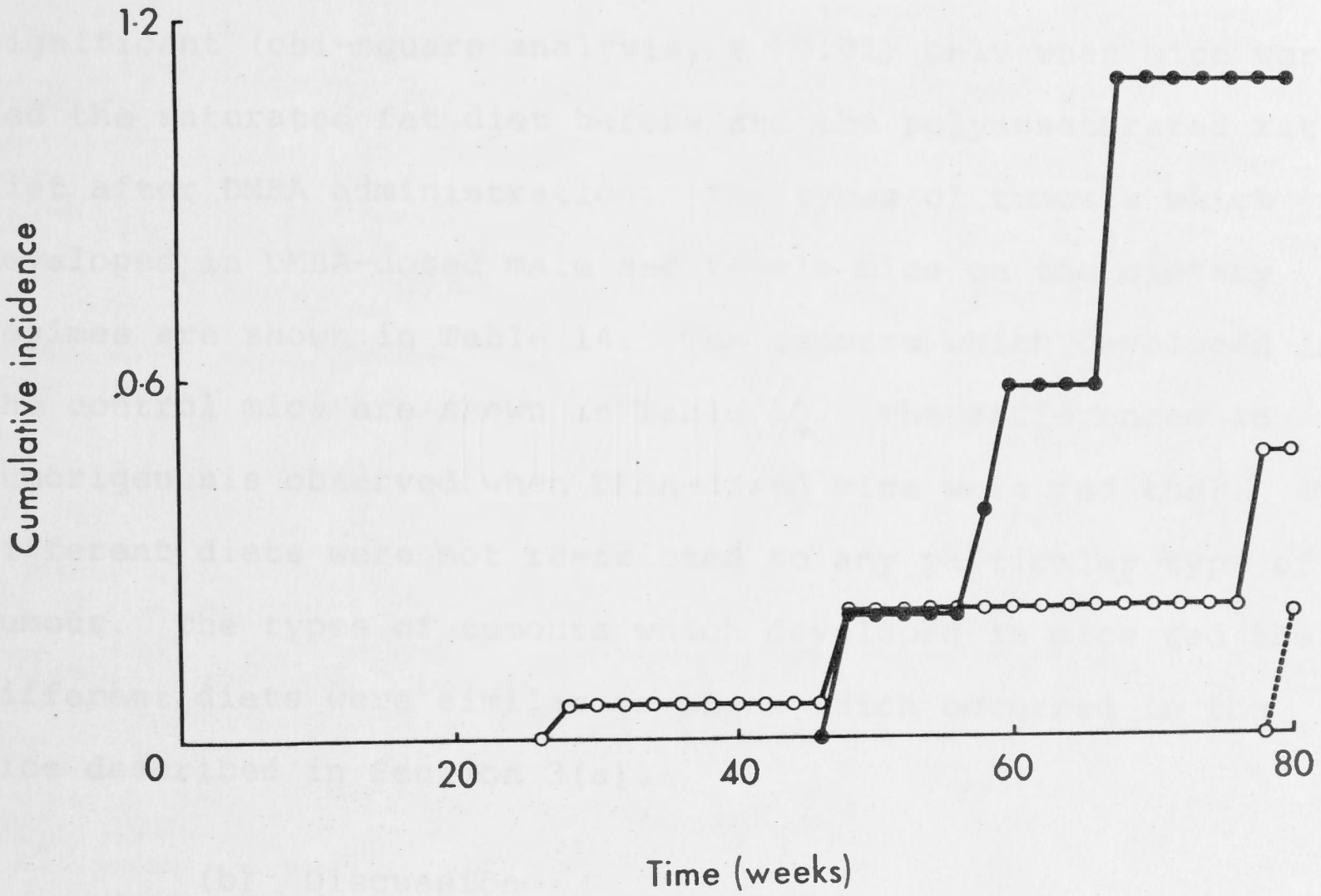


FIGURE 26. *Cumulative incidences of tumour-bearing male mice among DMBA-dosed mice fed the polyunsaturated fat diet throughout the experiment (●—●), and DMBA-dosed (○—○) and control (○- -○) mice fed the saturated fat diet throughout the experiment.* None of the control mice fed the polyunsaturated fat diet throughout the experiment developed tumours. The difference between the cumulative incidences among DMBA-dosed mice was not statistically significant.

The greater susceptibility of female mice to develop tumours, compared to male mice, described in Section 3(a) was confirmed in the present experiment. More DMBA-dosed female mice developed tumours than did DMBA-dosed male mice fed the same dietary regime. However, this difference was statistically significant (chi-square analysis, $P < 0.01$) only when mice were fed the saturated fat diet before and the polyunsaturated fat diet after DMBA administration. The types of tumours which developed in DMBA-dosed male and female mice on the dietary regimes are shown in Table 14. The tumours which developed in the control mice are shown in Table 15. The differences in tumorigenesis observed when DMBA-dosed mice were fed the different diets were not restricted to any particular type of tumour. The types of tumours which developed in mice fed the different diets were similar to those which occurred in the mice described in Section 3(a).

(b) Discussion

DMBA was shown to be toxic and to result in the death of mice which had received large or multiple doses of this compound and to cause decreased gains in body weight when mice received smaller doses. Such a reaction to DMBA was expected as it is known that DMBA causes adrenal apoplexy and massive necrosis in the inner zones of the adrenal cortex (Huggins and Morii, 1961). These authors did not observe haemorrhage and necrosis in other organs. Adrenal necrosis and apoplexy also presumably caused the early death of some of the DMBA-dosed rats described in Section 2. It has been suggested that the effect of DMBA on the adrenal gland is

TABLE 14. Types of tumours in DMBA-dosed mice fed the poly-unsaturated fat diet (P) or the saturated fat diet (S).

Tissue: type of tumour	Number of male mice with tumours — Dietary regime				Number of female mice with tumours — Dietary regime			
	PP[a]	SS	PS	SP	PP	SS	PS	SP
Colon: papilloma								1
Leukocyte-forming tissues:								
lymphoblastic leukaemia								1
lymphocytic leukaemia	1	1	1		1			
Liver: liver cell adenoma	2	2	3	3	3			2
hepatocellular carcinoma		1	1	3				
Lung: adenocarcinoma								1
adenoma	2			3	3			
Mammary gland:								
adenoacanthoma						1	1	
adenocarcinoma type A					2	1	5	2
adenocarcinoma type B					2	2	1	
adenocarcinoma mixed type A-type B								1
fibroma								1
Ovary: adenoma					1			
granulosa cell tumour					8	8	2	8
leiomyosarcoma							1	
mixed granulosa-thecal cell tumour					4			
mixed spindle cell-round cell tumour								1
Skin: basal cell carcinoma				1	1			
basosquamous cell carcinoma								2
papilloma					1			
squamous cell carcinoma	2			2		2		1
Stomach:								
squamous cell carcinoma				1	1			
Subcutaneous tissue:								
fibrosarcoma					2			
Uterus:								
fibroma					1	1		

[a]See footnote a on Table 13.

Some mice developed more than one tumour each.

TABLE 15. Types of tumours in control mice fed the poly-unsaturated fat diet (P) or the saturated fat diet (S).

Tissue: type of tumour	Number of male mice with tumours Dietary regime				Number of female mice with tumours Dietary regime			
	PP[a]	SS	PS	SP	PP	SS	PS	SP
Bone (pelvis): osteogenic sarcoma						1		
Leukocyte-forming tissues: lymphocytic leukaemia						1		
Liver: liver cell adenoma		1	1	1				
hepatocellular carcinoma					1	1		
Mammary gland: adenocarcinoma type B					1	1	1	
Ovary: granulosa cell tumour								1

[a] See footnote a on Table 13.

Most mice developed one tumour each. One female mouse in group SS developed an osteogenic sarcoma and a hepatocellular carcinoma.

related to the steric resemblance between DMBA and the corticosteroid hormones produced in the adrenal cortex (Morii and Huggins, 1962; Huggins *et al.*, 1963; Wong and Warner, 1964). This structural similarity could permit entry of DMBA into the adrenal cortex and a steric fit in molecular sites of the cortex where cortisone and hydrocortisone are synthesised. As DMBA has the ability to donate electrons to appropriate electron acceptors, charge-transfer complexes could be formed (Szent-Györgyi, 1960; Szent-Györgyi *et al.*, 1960). Thus once the DMBA is localised in a closely-fitted site, charge-transfer events could lead to the death of cells and to adrenal apoplexy around the damaged cells.

It is also possible that the necrotic effect of DMBA may be due to a disturbance of the corticosteroid content of the adrenal gland. As DMBA is oxidised preferentially at the side chain (Boyland and Sims, 1965) the hydroxymethyl derivative rather than the unchanged carcinogen may interfere with the metabolism or action of the corticosteroids. The presence of the hydroxymethyl group, characteristic of corticosteroids, would probably be a potent inhibitor of corticosteroid synthesis and the biochemical reactions influenced by these hormones (Jellinck and Goudy, 1967). Corticosteroids are known to stabilise lysosomal membranes and to inhibit the inflammatory response in small blood vessels after tissue injury (Weissmann and Thomas, 1964). A decrease in corticosteroid concentration may therefore render the membranes of the adrenal lysosomes exceptionally susceptible to damage by polycyclic hydrocarbons and the cytotoxic effects of DMBA could be due to release of lysosomal enzymes. It is also

known that polycyclic hydrocarbons are concentrated inside these organelles (Allison and Mallucci, 1964) and that the membranes of rat adrenal lysosomes, but not those of liver and kidney, are highly susceptible to damage by 7-hydroxy-methyl-12-methylbenz(*a*)anthracene (Allison and Dingle, 1966).

The types of tumours which developed in the DMBA-dosed C3HA^{vy}fB mice were more diverse than those reported by Heston and Vlahakis (1968) to occur spontaneously in C3HA^{vy} and C3HA^{vy}fB mice. These authors showed that the vast majority of tumours in these mice occurred in the liver and mammary glands. Both male and female mice developed liver tumours, but female mice died from mammary tumours at an earlier age. A few tumours also developed at sites such as the ovary, cervix, lung, adrenal gland and thymus. In the present experiments, mice which were not dosed with DMBA developed so-called spontaneous tumours at sites such as the liver, lung, mammary gland, ovary and leukocyte-forming tissues.

Most of the tumours which occurred in the DMBA-dosed mice in the present experiments have been shown to be induced by DMBA in other breeds of mice. Many of the previous experiments have used new-born mice whereas in the experiments described here the mice were approximately 98 days of age when the DMBA was administered. This raises a difficulty in the interpretation of the data because the relationship between the dose of carcinogen and the weight of some organs is not constant throughout life. The relative amount of carcinogen which reaches the target organ might therefore differ with age (Della Porta and Terracini, 1969). Nevertheless, DMBA

has been shown to induce tumours in the lymphoid tissues, primarily the thymus (Rask-Nielson, 1956; Pietra *et al.*, 1961; Roe *et al.*, 1961; Toth *et al.*, 1963; Gorrod *et al.*, 1968; Ball, 1970), in the lungs (Pietra *et al.*, 1961; Roe *et al.*, 1961; Toth *et al.*, 1963; Flaks, 1965; Walters and Roe, 1966; Gorrod *et al.*, 1968), in the skin (Terracini *et al.*, 1960; Turusov *et al.*, 1971), in the ovaries and mammary glands (Biancifiori *et al.*, 1961; Toth *et al.*, 1963; Medina, 1974), in the stomach (Biancifiori *et al.*, 1961; Toth *et al.*, 1963; Field and Roe, 1965) and in the liver, with the majority of liver tumours occurring in male mice (Roe *et al.*, 1961; Roe and Waters, 1967; Gorrod *et al.*, 1968).

The results of the experiment with mice fed the polyunsaturated and saturated fat diets support the conclusion from the work with rats (Section 2) that the polyunsaturated fat diet enhances DMBA-induced carcinogenesis. The diet appeared to exert its action after DMBA administration as mice which continued to receive the saturated fat diet throughout the experiment developed fewer tumours than those which were switched to the polyunsaturated fat diet after DMBA administration. Conversely, mice switched to the saturated fat diet after DMBA administration developed fewer tumours than those which received the polyunsaturated fat diet throughout the experiment. It is therefore likely that this enhancement of carcinogenesis involved the promotional stage of carcinogenesis rather than the initial event of neoplastic transformation. It is also probable that neoplastic transformation was complete before diet-induced changes had occurred in the

tissues of rats and mice fed a different diet after DMBA administration. This is likely because animals did not recommence the diets until 2 days after DMBA administration and because the diet-induced changes in the tissues would not have been immediate. Gammal *et al.* (1968) have shown that the maximum concentration of DMBA in the mammary tissue of rats which had received 10 mg DMBA intragastrically occurred within 12 hours of dosing. However, very small concentrations of DMBA did persist in the mammary tissue for at least 5 days.

In the present experiment the polyunsaturated fat diet appeared to be more effective in enhancing the tumour incidence when the mice were fed the diet for the first time after DMBA administration. The increase in the tumour incidence when female mice commenced the polyunsaturated fat diet after DMBA administration, compared to the incidence in the mice fed the saturated fat diet throughout the experiment, was greater than the increase when female mice were fed the polyunsaturated fat diet throughout the experiment. The level of statistical significance of the difference between the tumour incidences was also greater. A similar situation occurred with the DMBA-dosed rats described in Section 2. The increase in the tumour incidence when rats commenced the polyunsaturated fat diet after DMBA administration, compared to the incidence in the rats fed the saturated fat diet throughout the experiment, was greater than the increase when rats were fed the polyunsaturated fat diet throughout the experiment. The former difference between the tumour incidences was statistically significant, while the latter was not significant. The reason for the

greater effect of the polyunsaturated fat diet when given for the first time after DMBA administration is not known. It is possible that when the animals commenced the diet the concentration in the tissues of the dietary factors enhancing carcinogenesis increased. Over a period of time homeostatic mechanisms may have partly restored the normal internal environment of the animals.

Mechanisms possibly responsible for the enhancement of carcinogenesis in animals fed the polyunsaturated fat diet have been discussed in Section 2. In addition to those mentioned previously it is possible that the large amounts of linoleic acid in the polyunsaturated fat diet could have enabled greater rates of membrane synthesis in neoplastic cells of animals fed this diet. Hillyard and Abraham (1972) have shown that the rate of synthesis of phosphatidylcholine, a membrane constituent, is correlated with the rate of cellular proliferation. Since the synthesis of phospholipids and hence new membranes requires linoleic acid, which is an essential fatty acid, it is possible that the availability of linoleic acid could have been important in determining the rate of proliferation of neoplastic cells in the DMBA-dosed animals.

4. Growth of a transplantable adenocarcinoma in mice receiving different dietary fats

The experiments described in Sections 2 and 3 show that the susceptibility of rats and mice to develop DMBA-induced tumours was enhanced when the animals were fed the polyunsaturated fat diet rather than the saturated fat diet. This enhancement of carcinogenesis occurred only when the animals were fed the polyunsaturated fat diet after administration of the carcinogen. It is likely therefore that the effect of the polyunsaturated fat diet was exerted on the promotional stage of carcinogenesis rather than on the initial event of neoplastic transformation. The promotional stage of carcinogenesis involves the proliferation of neoplastic cells to form an established tumour (Berenblum, 1954). The present experiment was designed therefore to examine the effect of the high fat diets on the growth of tumour cells into discrete tumours. The incidence and induction times of tumours have been examined in mice inoculated with a suspension of single cells prepared from a transplantable mammary adenocarcinoma and fed either the polyunsaturated or the saturated fat diet.

(a) Results

Female mice fed the low fat diet were used to determine the number of tumour cells required to produce palpable tumours in 50% of the animals inoculated (TD 50). The proportions of mice developing tumours within 42 days after inoculation with various numbers of tumour cells are shown in Figure 27. None of the mice in the experiments described in this Section developed tumours after this 42 day

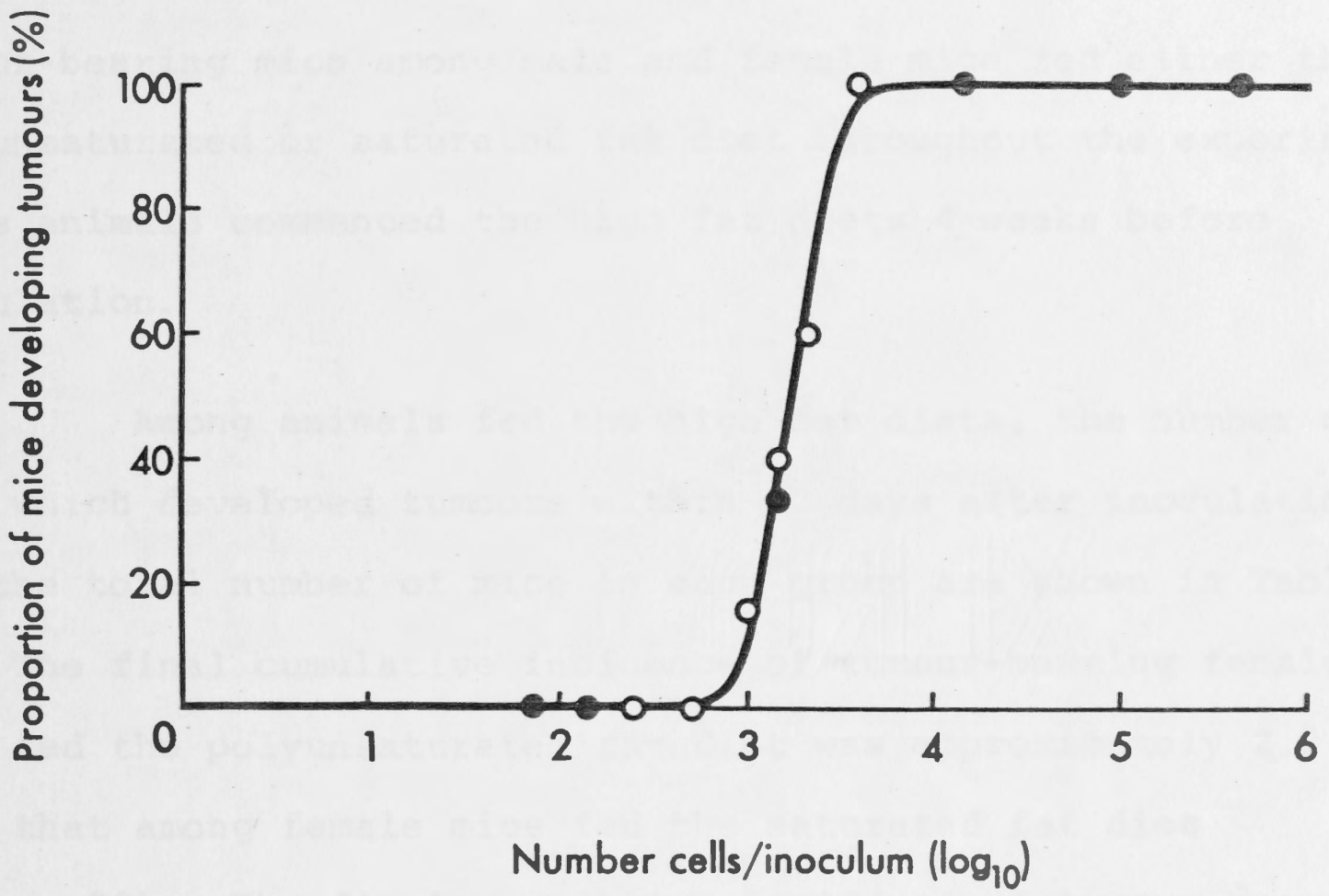


FIGURE 27. *Proportion of female mice developing palpable tumours after subcutaneous inoculation with various numbers of viable cells from a transplantable mammary adenocarcinoma.* These mice were fed the low fat diet. Experiment 1, ●—● ; experiment 2, O—O .

Each point represents data from 10 mice.

period. The TD 50 value calculated by the method of Reed and Muench (1938) was approximately 1,700 cells. Inocula of this number of tumour cells were used to examine the incidence of tumour-bearing mice among male and female mice fed either the polyunsaturated or saturated fat diet throughout the experiment. These animals commenced the high fat diets 4 weeks before inoculation.

Among animals fed the high fat diets, the number of mice which developed tumours within 42 days after inoculation and the total number of mice in each group are shown in Table 16. The final cumulative incidence of tumour-bearing female mice fed the polyunsaturated fat diet was approximately 2.2 fold that among female mice fed the saturated fat diet (Figure 28). The final cumulative incidence of tumour-bearing male mice fed the polyunsaturated fat diet was approximately 2.7 fold that among male mice fed the saturated fat diet (Figure 29). Both these differences were statistically significant (chi-square analysis: female mice, $P < 0.025$; male mice, $P < 0.05$). It is also interesting that the female mice were more susceptible to the development of tumours than the male mice. The differences in cumulative tumour incidence between male and female mice fed the same diet were statistically significant (chi-square analysis: polyunsaturated fat diet, $P < 0.001$; saturated fat diet, $P < 0.01$). The mean induction times of tumours decreased when mice were fed the polyunsaturated fat diet, but these differences were not statistically significant (Table 17).

TABLE 16. Number of tumour-bearing mice among animals inoculated with a tumour cell suspension and fed either the polyunsaturated or saturated fat diet.

Sex	Diet	Time after inoculation, days													
		1-3	4-6	7-9	10-12	13-15	16-18	19-21	22-24	25-27	28-30	31-33	34-36	37-39	40-42
Female	Polyunsaturated fat	0/28[a]	0/28	0/28	2/28	5/26	4/21	0/17	4/17	5/13	3/8	0/5	1/5	0/4	0/4[b]
	Saturated fat	0/27	0/27	0/27	0/27	0/27	1/27	3/26	5/23	2/18	3/16	0/13	0/13	1/13	0/12
Male	Polyunsaturated fat	0/32	0/32	0/32	0/32	0/32	1/32	1/31	6/30	3/24	1/21	2/20	1/18	0/17	0/17
	Saturated fat	0/30	0/30	0/30	0/30	0/30	1/30	0/29	0/29	3/29	1/26	0/25	0/25	1/25	0/24

[a] x/y: x is the number of mice developing palpable tumours during each 3 day period.
y is the number of mice without tumours at the beginning of each 3 day period.

[b] Mice did not develop tumours after day 42.

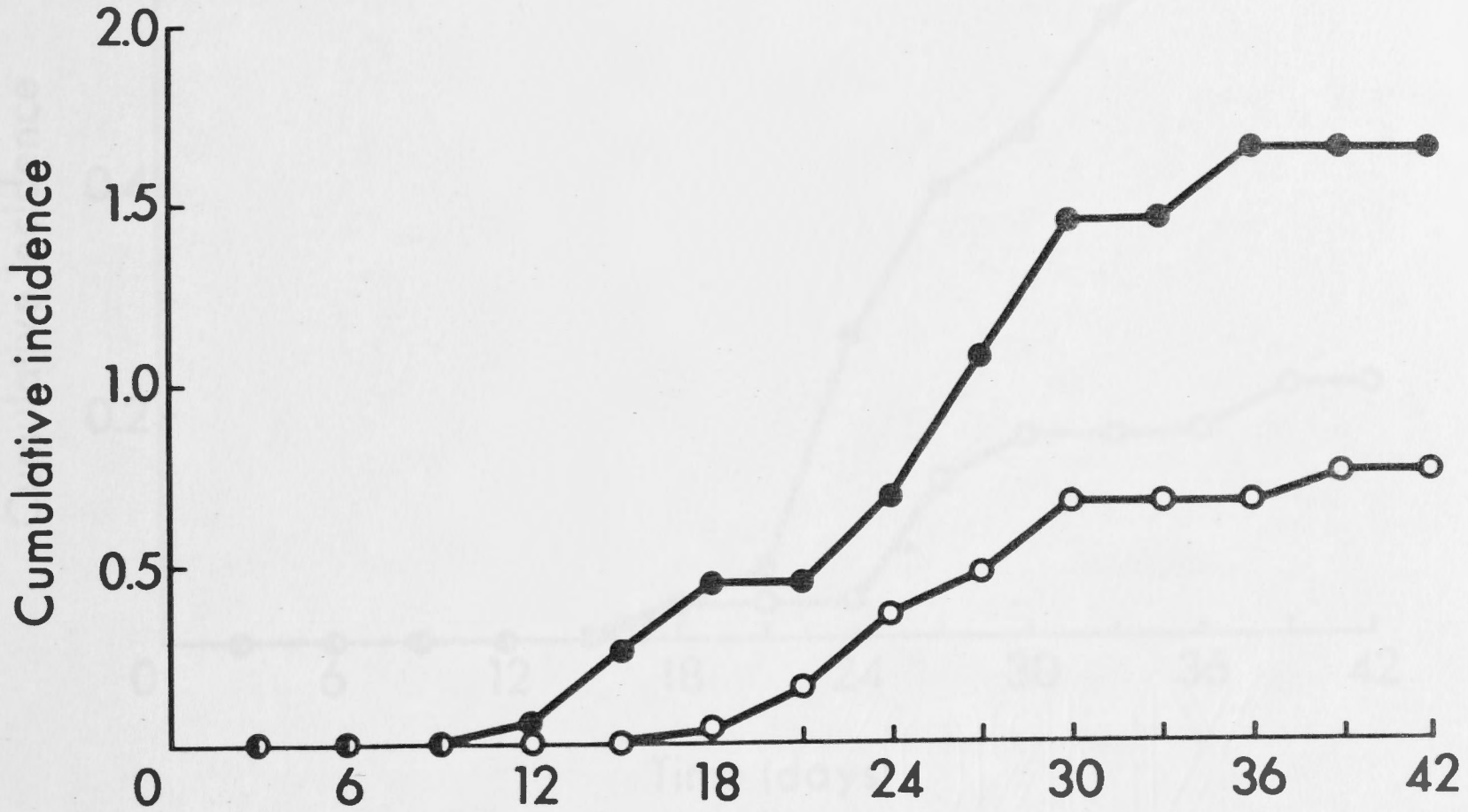


FIGURE 28. *Cumulative incidence of tumour-bearing mice among female mice inoculated with approximately 1,700 viable cells from a transplantable mammary adenocarcinoma and fed either the polyunsaturated fat diet (●—●) or the saturated fat diet (○—○).* The difference between the cumulative incidences was statistically significant (chi-square analysis: $P < 0.025$). In this experiment the mice were inoculated on day zero.

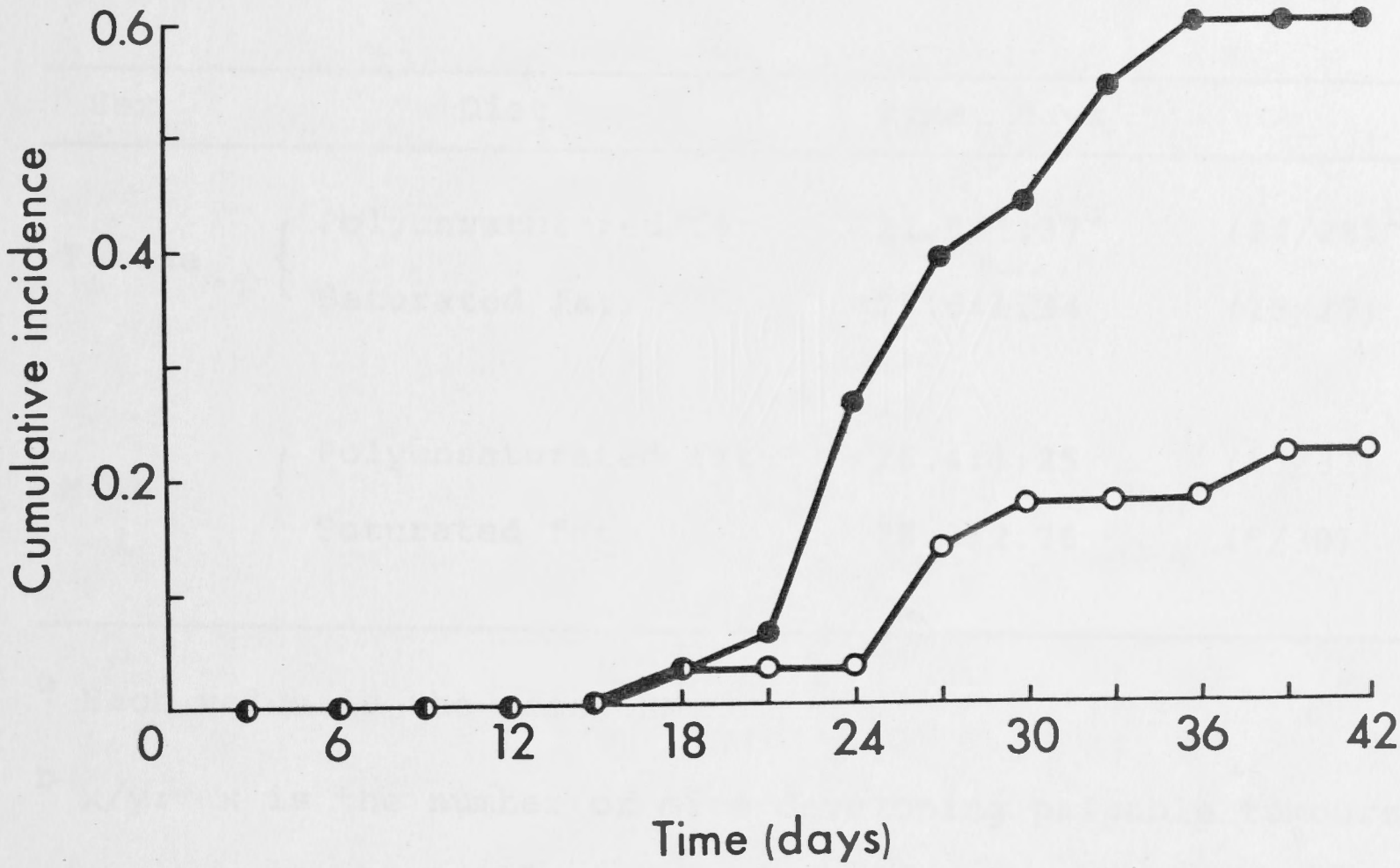


FIGURE 29. *Cumulative incidence of tumour-bearing mice among male mice inoculated with approximately 1,700 viable cells from a transplantable mammary adenocarcinoma and fed either the polyunsaturated fat diet (●—●) or the saturated fat diet (○—○).* The difference between the cumulative incidences was statistically significant (chi-square analysis: $P < 0.05$).

TABLE 17. Induction times of palpable tumours in mice inoculated with a tumour cell suspension and fed either the polyunsaturated or saturated fat diet.

Sex	Diet	Time, days	
Female	Polyunsaturated fat	22.0±1.37[a]	(24/28)[b]
	Saturated fat	25.6±1.34	(15/27)
Male	Polyunsaturated fat	26.4±1.25	(15/32)
	Saturated fat	28.0±2.76	(6/30)

[a] Each value is the mean±SEM.

[b] x/y: x is the number of mice developing palpable tumours.
y is the total number of mice.

(b) Discussion

Mice fed a diet containing polyunsaturated fat were more susceptible to the development of tumours following inoculation with cells from a transplantable adenocarcinoma than were mice fed a diet containing more saturated fat. The differences in cumulative tumour incidence therefore support the suggestion made in Sections 2 and 3 that the enhancement of carcinogenesis by dietary polyunsaturated fat is exerted on the promotional stage of carcinogenesis.

Taken alone, the statistically significant differences in induction times of DMBA-induced tumours in the rats fed the different dietary regimes (Section 2) and the lack of statistically significant differences in induction times in the present experiment could imply that the dietary fat exerts its effect on the initiation stage of carcinogenesis. However, the experiments with DMBA-induced tumours (Sections 2 and 3) have shown that this is not the case.

Recent experiments by Rao and Abraham (1976) have also shown that tumour growth is enhanced in mice fed a diet containing polyunsaturated fat. The growth of transplanted pieces of mammary adenocarcinoma was greater in mice fed diets containing various amounts of corn oil than in mice fed a fat free diet or a diet containing hydrogenated cotton-seed oil. These workers also determined the fatty acid content and the fatty acid composition of the tumour lipids in mice fed the diets. While the concentrations of total fatty acids were similar under all the dietary conditions there were differences in the fatty acid composition. Tumour lipids in mice fed diets containing increased amounts of corn oil contained progressively greater proportions of linoleic acid. This was in contrast to the proportion of arachidonic acid which reached a maximum when mice were fed the diet containing the least amount of corn oil. The effect of the corn oil diets on the growth of tumours parallelled the changes in the proportion of arachidonic acid in the tumour lipids rather than the changes in the proportion of linoleic acid. It is possible therefore that if tumour lipids are important in determining the growth of tumours the proportion of arachidonic acid, rather than

linoleic acid, is likely to be of consequence.

As discussed in the Introduction, the endocrine environment is important in determining the growth of rodent mammary tumours in that both oestrogen and prolactin are necessary for continued tumour growth. Therefore, differences in hormonal status could have been responsible for the differences in tumour incidence found in the present experiment. In particular, this system is likely to have been of importance in determining the greater tumour incidence in female mice than in male mice fed the same diet. The experiments with DMBA-dosed mice (Section 3) have shown similar differences in tumour-incidence between male and female mice.

5. Diet-induced changes in the fatty acid composition of mouse hepatocyte plasma membranes

The role of plasma membranes in carcinogenesis has been recognised for some time (Coman, 1953; Huxley, 1958; Wallach, 1972) and evidence now exists that neoplasms contain plasma membrane variants. Altered membrane structure could cause morphological changes, produce new antigenicity, alter binding of hormones and metabolites, modify the function of membrane-associated enzymes and change cell permeabilities. Although a unifying hypothesis explaining the role of the plasma membrane has not yet emerged, one possibility is that alterations in membrane structure could alter the transport of critical nutrients, thus conferring selective advantage to neoplastic cells in a physiologically competitive environment. (Wallach, 1972).

The simplest method of controlling growth and metabolism *in vitro* is to limit the supply of an essential nutrient such as an amino acid, a vitamin, oxygen or glucose. Specific nutrients such as glutamine and isoleucine (Ley and Tobey, 1970; Enger and Tobey, 1972), zinc (Lieberman and Ove, 1962; Rubin, 1972) and putrescine (Pohjanpelto and Raina, 1972) can regulate cell growth under certain conditions. Deficiencies of these nutrients can also arrest cell growth in the G1 phase of the cell cycle. The growth and metabolism of cells *in vivo* could also be controlled by factors derived from the serum. Serum contains a complex mixture of growth factors (Temin, 1968; Lipton *et al.*, 1971; Paul *et al.*, 1971; Wolstenholm and Knight, 1971) and some of these factors affect the uptake of nutrients by cells (Cunningham and Pardee, 1969; Hershko

et al., 1971; Sefton and Rubin, 1971). Since the concentrations of low molecular weight nutrients in blood are relatively constant throughout an animal, it has been suggested by Holley (1972) that cells control their growth rates by selectively altering the availability of nutrients to the interior of the cell. This could be regulated by transport systems in the plasma membrane of the cell or by structural changes in the cell membrane that increase permeability.

Studies with liposomes (de Gier *et al.*, 1968; Demel *et al.*, 1968; de Gier *et al.*, 1970; McElhaney *et al.*, 1970; Klein *et al.*, 1971; Demel *et al.*, 1972) and bacteria (McElhaney *et al.*, 1970; van der Neut-Kok, 1974) have shown that membranes containing unsaturated lipids are more permeable than those containing more saturated lipids. It was therefore of interest to examine the changes induced by diet in the fatty acid composition of plasma membranes. In the present experiments the fatty acid compositions of hepatocyte plasma membrane lipids from mice transferred from the low fat diet to the poly-unsaturated and saturated fat diets have been determined.

(a) Results

(i) Isolation of plasma membranes

A typical sedimentation profile obtained by rate-isopycnic centrifugation is shown in Figure 30. Plasma membranes were collected in a sharp peak with an average banding density from 3 runs of 1.18 g/ml at 20°C. This procedure resulted in a 31 fold increase in the specific activity, on a protein weight basis, of 5'-nucleotidase compared to that in a 10% homogenate of liver. The yield of

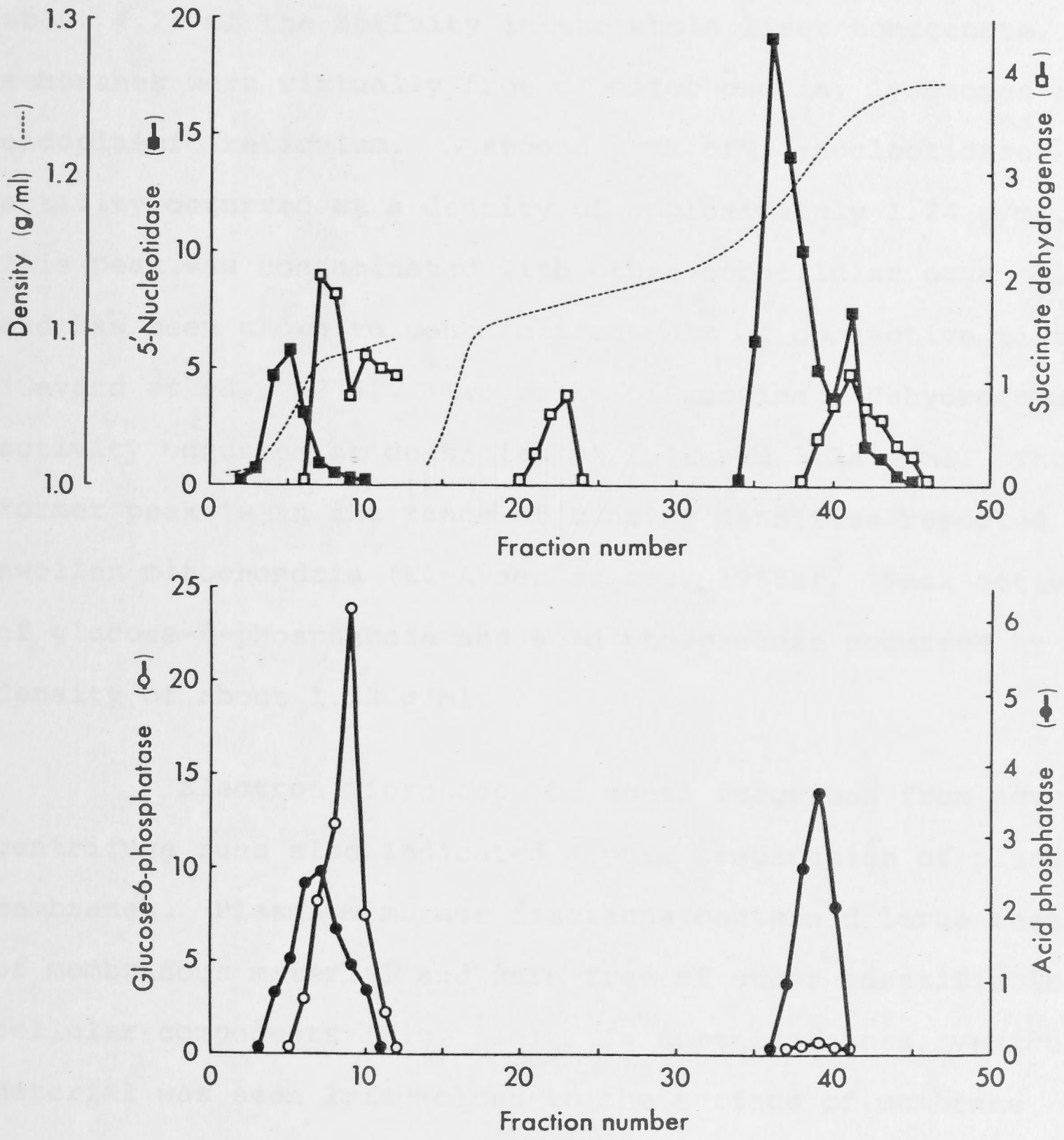


FIGURE 30. *Rate-isopycnic zonal fractionation of hepatic nuclear sediment.* The activities of enzymes are expressed as follows: 5'-nucleotidase and glucose-6-phosphatase, μmoles inorganic phosphate released per 14 ml zonal fraction per h; succinate dehydrogenase, μmoles 2,6-dichlorophenolindophenol reduced per 14 ml zonal fraction per h; acid phosphatase, μmoles *p*-nitrophenol reduced per 14 ml zonal fraction per h.

5'-nucleotidase activity in the plasma membrane fraction was about 4.2% of the activity in the whole liver homogenate. The membranes were virtually free of mitochondria, lysosomes and endoplasmic reticulum. A second peak of 5'-nucleotidase activity occurred at a density of approximately 1.24 g/ml. This peak was contaminated with other subcellular organelles and has been shown to contain fragments of connective tissue (Gavard *et al.*, 1974). Two peaks of succinate dehydrogenase activity occurred at densities of 1.12 and 1.24 g/ml. The former peak is in the range of banding densities reported for swollen mitochondria (El-Aaser *et al.*, 1966a). Peak activities of glucose-6-phosphatase and acid phosphatase occurred at a density of about 1.22 g/ml.

Electron microscopy of zonal fractions from several centrifuge runs also indicated a pure preparation of plasma membranes. Plasma membrane fractions contained large sheets of membranous material and were free of other identifiable cellular components (Fig. 31a). In some instances, vesicular material was seen lying close to the surface of membrane sheets (Fig. 31b). These vesicles have been reported to be microvilli or pinocytotic vesicles attached to the membrane sheet (Gavard *et al.*, 1974), but may also represent disrupted bile spaces or vesicular remnants of smooth endoplasmic reticulum which might be continuous with the plasma membrane (Benedetti & Emmelot, 1968).

(ii) Fatty acid composition of hepatocyte plasma membranes

The fatty acid composition of hepatocyte plasma membrane fractions prepared from female and male mice in the

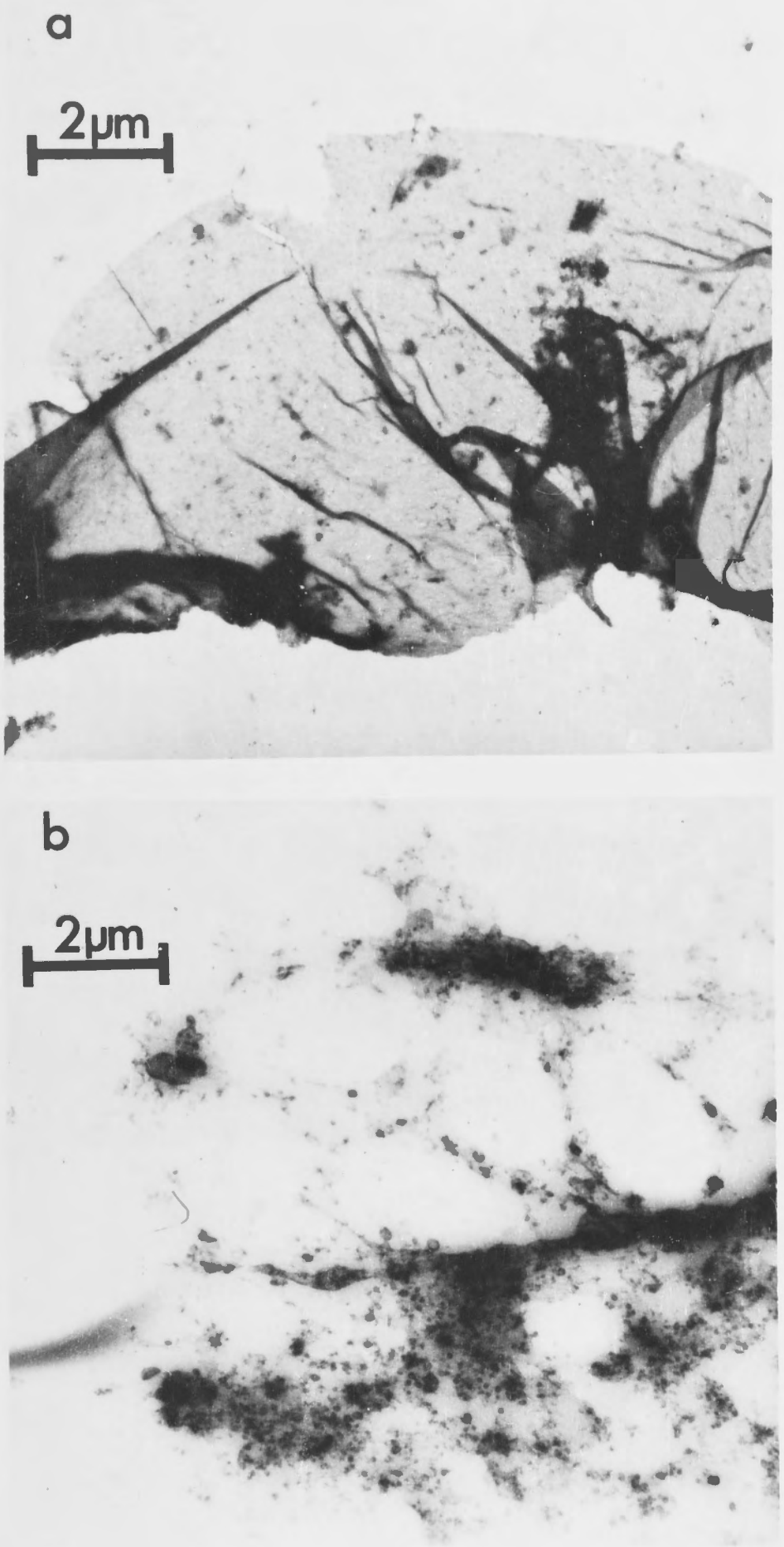


FIGURE 31. *Electron micrographs of plasma membranes from a zonal fraction.* a. Typical plasma membrane sheet, magnification 8,500X. b. Vesicular material associated with plasma membrane sheet, magnification 8,500X. Samples of zonal fractions were spread on copper grids (mesh size 150) which had been coated with collodion in iso-amyl acetate (3% w/v), were negatively stained with phosphotungstic acid (1% w/v) and neutralised with 0.1N NaOH.

first experiment are shown in Tables 18 and 19 respectively. There was some variation in the proportions of individual fatty acids in the plasma membrane lipids prepared from different mice. The fatty acid compositions of the plasma membrane lipids from male and female mice fed the low fat diet were similar, with palmitic and stearic acids constituting over 70% of the fatty acids. The major unsaturated fatty acids were oleic, linoleic and arachidonic acids. In order to substantiate the results of the first experiment the effects of the diets on the fatty acid composition of hepatocyte plasma membrane lipids was examined in a second experiment. The mice in this experiment were fed either of the high fat diets after the low fat diet, while in the first experiment mice were fed the same dietary regimes as were the DMBA-dosed rats and mice (Sections 2 and 3). The results for female and male mice in the second experiment are shown in Tables 20 and 21, respectively. The fatty acid compositions of plasma membrane lipids from male and female mice fed the low fat diet were similar. With the exception of a small proportion of lauric acid in the plasma membrane lipids of mice in the first experiment, the same fatty acids were present in hepatocyte plasma membrane lipids from mice fed the low fat diet in both experiments. There were, however, differences in the proportions of several fatty acids. In the second experiment, the plasma membrane lipids of mice fed the low fat diet contained greater proportions of oleic and linoleic acids, and smaller proportions of stearic acid than in the first experiment.

In the first experiment the plasma membrane lipids of mice fed the polyunsaturated fat diet for 4 weeks

TABLE 18. Fatty acid composition of hepatocyte plasma membranes from female mice fed the low fat diet or the high fat diets containing either polyunsaturated or saturated fat.

Fatty acids	Diet: Low fat	Polyunsaturated fat		Saturated fat		
	Time (weeks)					
	0	2	4	6	8	30
12:0[a]	1.1±0.43[b]					
14:0	9.8±0.18	1.4±0.03	2.8±0.03	1.6±0.03	2.0±0.07	1.3±0.29
16:0	27.8±0.85	39.7±0.03	36.4±0.61	42.7±0.15	40.5±0.50	32.0±0.47
16:1	1.3±0.03	Trace[c]		1.1±0.03	2.1±0.03	2.3±0.53
18:0	42.8±0.78	49.4±0.22	33.9±0.61	25.7±0.12	25.6±0.32	21.9±0.55
18:1	4.9±0.10	1.2±0.03	5.8±0.12	10.5±0.07	17.3±0.27	30.6±0.54
18:2	1.9±0.15	2.1±0.06	6.5±0.37	5.1±0.21	2.1±0.09	3.3±0.22
18:3			1.3±0.40	Trace		
20:0	6.6±0.03	2.8±0.03	Trace	1.0±0.07	1.1±0.03	Trace
20:2				0.9±0.03	1.0±0.04	
20:4	3.8±0.15	3.4±0.03	6.8±0.94	6.0±0.78	4.8±0.38	8.6±1.75
22:0			3.9±0.56	3.6±1.06	3.5±0.38	
22:2			2.6±0.65	1.8±0.32		
Saturates	88.1	93.3	77.0	74.6	72.7	55.2
Monoenes	6.2	1.2	5.8	11.6	19.4	32.9
Dienes	1.9	2.1	9.1	7.8	3.1	3.3
Polyenes[d]	3.8	3.4	8.1	6.0	4.8	8.6
Mean chain length[e]	17.2	17.3	17.6	17.4	17.4	17.4
Unsaturation index[f]	25.2	19.0	55.1	51.2	44.8	73.9

Fatty acids	Diet: Low fat	Saturated fat		Polyunsaturated fat		
	Time (weeks)					
	0	2	4	6	8	30
12:0	1.1±0.43					
14:0	9.8±0.18	4.1±0.21	0.9±0.07	2.6±0.09	1.9±0.04	4.0±0.03
16:0	27.8±0.85	24.2±0.03	34.6±0.32	30.2±0.69	20.8±1.15	44.5±0.15
16:1	1.3±0.03	1.7±0.06	2.0±0.03	4.2±0.92	Trace	1.0±0.03
18:0	42.8±0.78	42.4±1.30	35.3±0.24	28.8±0.64	44.0±0.55	29.4±0.12
18:1	4.9±0.10	16.2±0.47	21.5±0.59	20.7±0.46	10.2±0.70	15.0±0.06
18:2	1.9±0.15	3.5±0.34	2.3±0.12	5.0±0.21	11.8±0.80	2.7±0.20
18:3				1.7±0.06	1.3±0.06	
20:0	6.6±0.03	4.5±0.28	1.0±0.03	2.3±0.51	1.2±0.05	1.0±0.40
20:2				Trace	1.8±0.10	
20:4	3.8±0.15	3.4±0.06	2.4±0.12	3.4±0.32	7.0±0.25	2.4±0.10
22:0				1.1±0.29		Trace
Saturates	88.1	75.2	71.8	65.0	67.9	78.9
Monoenes	6.2	17.9	23.5	24.9	10.2	16.0
Dienes	1.9	3.5	2.3	5.0	13.6	2.7
Polyenes	3.8	3.4	2.4	5.1	8.3	2.4
Mean chain length	17.2	17.5	17.3	17.4	17.7	17.0
Unsaturation index	25.2	38.5	37.7	53.6	69.3	31.0

Each value is the mean±SEM of 3 determinations on a pooled sample of liver from 2 or 3 mice.

[a] Number of C atoms : number of double bonds.

[b] Units are mole % of total fatty acids.

[c] < 0.5%.

[d] 3 or more double bonds.

[e] Mean number of C atoms per fatty acid.

[f] Sum of the products of mole % and the number of double bonds per fatty acid. The index divided by 100 gives the number of double bonds per fatty acid molecule (White *et al.*, 1971).

TABLE 19. Fatty acid composition of hepatocyte plasma membranes from male mice fed the low fat diet or the high fat diets containing either polyunsaturated or saturated fat.

Fatty acids	Diet: Low fat	Polyunsaturated fat		Saturated fat		
	Time (weeks)					
	0	2	4	6	8	30
12:0[a]	1.2±0.13[b]	1.4±0.12				
14:0	5.6±0.13	1.5±0.06	2.6±0.12	0.9±0.05	1.0±0.09	1.9±0.12
16:0	25.8±0.64	25.8±0.72	24.0±0.95	31.8±0.55	25.1±2.18	23.5±0.51
16:1	1.7±0.03	2.3±0.06	1.4±0.07	0.5±0.05	4.8±0.47	3.6±0.37
18:0	47.0±0.68	46.3±1.45	42.2±0.15	30.7±0.60	34.3±3.65	20.1±1.07
18:1	4.7±0.11	5.8±0.20	6.1±0.05	21.7±0.25	17.1±1.97	31.2±2.27
18:2	2.2±0.06	2.7±0.35	10.1±0.15	8.1±0.32	6.5±1.37	3.8±0.18
18:3			5.5±0.60	1.2±0.06		
20:0	6.2±0.13	8.2±0.52	1.1±0.25	1.0±0.05	6.6±1.32	6.8±2.94
20:2						1.6±0.54
20:4	5.6±0.47	6.0±0.43	7.0±0.25	4.1±0.07	4.6±1.73	7.5±0.38
22:0			Trace[c]			
Saturates	85.8	83.2	69.9	64.4	67.0	52.3
Monoenes	6.4	8.1	7.5	22.2	21.9	34.8
Dienes	2.2	2.7	10.1	8.1	6.5	5.4
Polyenes[d]	5.6	6.0	12.5	5.3	4.6	7.5
Mean chain length[e]	17.4	17.6	17.6	17.4	17.6	17.7
Unsaturation index[f]	33.2	37.5	72.2	58.4	53.3	75.6

Fatty acids	Diet: Low fat	Saturated fat		Polyunsaturated fat		
	Time (weeks)					
	0	2	4	6	8	30
12:0	1.2±0.13					
14:0	5.6±0.13	1.4±0.06	1.1±0.03	0.8±0.07	1.4±0.03	1.7±0.10
16:0	25.8±0.64	43.9±0.90	43.4±0.42	45.1±1.25	34.1±0.24	36.1±0.25
16:1	1.7±0.03	0.6±0.05	Trace	0.7±0.05	Trace	Trace
18:0	47.0±0.68	43.8±0.85	43.8±0.39	41.2±1.12	30.2±0.24	29.8±0.20
18:1	4.7±0.11	4.5±0.12	7.7±0.07	6.9±0.15	15.5±0.15	23.3±0.27
18:2	2.2±0.06	2.0±0.05	1.7±0.03	2.8±0.05	9.7±0.12	4.1±0.60
18:3				Trace	1.6±0.48	Trace
20:0	6.2±0.13			Trace	1.2±0.15	1.7±0.25
20:4	5.6±0.47	3.8±0.17	2.3±0.23	2.5±0.33	6.3±0.03	3.3±0.10
Saturates	85.8	89.1	88.3	87.1	66.9	69.3
Monoenes	6.4	5.1	7.7	7.6	15.5	23.3
Dienes	2.2	2.0	1.7	2.8	9.7	4.1
Polyenes	5.6	3.8	2.3	2.5	7.9	3.3
Mean chain length	17.4	17.1	17.1	17.1	17.4	17.3
Unsaturation index	33.2	24.3	20.3	23.2	64.9	44.7

Each value is the mean±SEM of 3 determinations on a pooled sample of liver from 2 or 3 mice.

a,b,c,d,e,f See footnotes of Table 18.

TABLE 20. Fatty acid composition of hepatocyte plasma membranes from female mice fed the low fat diet or the high fat diets containing either polyunsaturated or saturated fat.

Fatty acids	Diet: Low fat	Diet: Polyunsaturated fat		
	Time (weeks)			
	0	2	4	8
14:0[a]	9.6±0.10[b]	6.1±0.09	2.3±0.03	4.7±0.09
16:0	31.4±0.23	25.3±0.35	19.9±0.38	32.8±0.29
16:1	1.3±0.06	1.0±0.03	3.7±0.59	0.9±0.15
18:0	26.7±1.79	29.4±0.41	29.9±0.64	38.3±0.35
18:1	12.3±1.05	10.4±0.20	8.0±1.04	8.1±0.26
18:2	5.1±0.48	7.5±0.27	11.8±0.74	3.2±0.09
18:3		Trace[c]	1.7±0.23	
20:0	7.2±1.94	11.4±0.09	6.6±1.35	5.0±0.18
20:2		2.4±0.15	2.7±0.06	0.9±0.09
20:4	6.4±2.01	6.5±0.10	8.8±0.52	6.1±0.49
22:0			2.9±0.10	
22:2			1.7±0.42	
Saturates	74.9	72.2	61.6	80.8
Monoenes	13.6	11.4	11.7	9.0
Dienes	5.1	9.9	16.2	4.1
Polyenes[d]	6.4	6.5	10.5	6.1
Mean chain length[e]	17.2	17.6	18.0	17.4
Unsaturation index[f]	49.4	57.2	84.4	41.6

Fatty acids	Diet: Low fat	Diet: Saturated fat		
	Time (weeks)			
	0	2	4	8
14:0	9.6±0.10	2.5±0.17	2.7±0.03	3.9±0.06
16:0	31.4±0.23	29.3±2.43	30.3±0.54	36.7±0.52
16:1	1.3±0.06	1.3±0.21	2.4±0.25	1.3±0.03
18.0	26.7±1.79	26.8±0.55	25.3±0.19	28.9±0.65
18:1	12.3±1.05	19.6±0.72	20.8±0.29	15.5±0.07
18:2	5.1±0.48	5.4±0.61	5.5±0.10	2.0±0.15
20:0	7.2±1.92	8.2±0.73	2.2±0.03	4.6±0.09
20:2			0.8±0.12	
20:4	6.4±2.01	6.9±0.12	6.7±0.86	5.0±0.95
22:0			3.3±0.06	2.1±0.54
Saturates	74.9	66.8	63.8	76.2
Monoenes	13.6	20.9	23.2	16.8
Dienes	5.1	5.4	6.3	2.0
Polyenes	6.4	6.9	6.7	5.0
Mean chain length	17.2	17.6	17.6	17.4
Unsaturation index	49.4	59.3	62.6	40.8

Each value is the mean±SEM of 3 determinations on a pooled sample of liver from 2 or 3 mice.

a,b,c,d,e,f See footnotes of Table 18.

TABLE 21. Fatty acid composition of hepatocyte plasma membranes from male mice fed the low fat diet or the high fat diets containing either polyunsaturated or saturated fat.

Fatty acids	Diet: Low fat	Diet: Polyunsaturated fat		
	Time (weeks)			
	0	2	4	8
14:0[a]	10.5±0.05[b]	5.9±0.33	0.9±0.03	3.3±0.03
16:0	31.3±0.15	24.2±1.35	24.0±0.29	32.2±0.35
16:1	1.2±0.05	1.5±0.47	1.5±0.35	0.9±0.07
18:0	23.3±0.45	32.8±0.38	32.6±0.17	42.2±0.50
18:1	12.3±0.30	8.3±0.25	8.5±0.36	9.3±0.12
18:2	8.8±0.20	9.6±0.48	16.5±0.18	4.8±0.07
18:3			2.3±0.23	
20:0	7.5±0.05	7.5±0.18	1.4±0.23	3.4±0.09
20:2		1.8±0.35	1.5±0.26	Trace[c]
20:4	5.1±1.05	4.9±0.43	8.3±0.21	3.9±0.98
22:0		3.5±0.47	2.5±0.09	
Saturates	72.6	73.9	61.4	81.1
Monoenes	13.5	9.8	10.0	10.2
Dienes	8.8	11.4	18.0	4.8
Polyenes[d]	5.1	4.9	10.6	3.9
Mean chain length[e]	17.2	17.7	17.8	17.4
Unsaturation index[f]	51.5	52.2	86.1	35.4

Fatty acids	Diet: Low fat	Diet: Saturated fat		
	Time (weeks)			
	0	2	4	8
14:0	10.5±0.05	3.2±0.12	4.8±0.19	4.1±0.07
16:0	31.3±0.15	24.6±0.95	30.0±1.01	35.7±0.60
16:1	1.2±0.05	4.9±0.23	2.1±0.27	1.3±0.03
18:0	23.3±0.45	28.8±0.37	28.2±1.08	30.8±0.15
18:1	12.3±0.30	11.3±1.12	15.4±0.09	15.8±0.91
18:2	8.8±0.20	8.3±0.80	7.3±0.94	2.2±0.09
20:0	7.5±0.05	7.2±1.01	6.4±0.32	4.5±0.09
20:2		2.0±0.06		
20:4	5.1±1.05	6.0±0.74	5.8±1.92	3.6±0.53
22:0		3.7±0.17		2.0±0.30
Saturates	72.6	67.5	69.4	77.1
Monoenes	13.5	16.2	17.5	17.1
Dienes	8.8	10.3	7.3	2.2
Polyenes	5.1	6.0	5.8	3.6
Mean chain length	17.2	17.7	17.4	17.3
Unsaturation index	51.5	60.8	55.3	35.9

Each value is the mean±SEM of 3 determinations on a pooled sample of liver from 2 or 3 mice.

[a,b,c,d,e,f] See footnotes of Table 18.

contained increased proportions of the major dietary fatty acid, linoleic acid, and increased proportions of arachidonic acid. The proportions of these fatty acids declined when mice continued to receive the polyunsaturated fat diet. These findings were confirmed in the second experiment. In this experiment the proportions of linoleic and arachidonic acids reached a maximum after mice had been fed the polyunsaturated fat diets for 4 weeks, but had declined after mice had received the diet for 8 weeks. When mice were fed the saturated fat diet after the polyunsaturated fat diet, the proportion of linoleic acid also declined. The proportion of oleic acid in the plasma membrane lipids increased when mice in the first experiment were fed either of the high fat diets. In the second experiment this increase was observed only when mice were fed the saturated fat diet, possibly because of the shorter time scale of the second experiment and the higher proportion of oleic acid in the saturated fat diet than in the polyunsaturated fat diet.

The mean carbon chain lengths and molar unsaturation indices of the fatty acids present in the plasma membrane lipids from female and male mice in the first experiment are shown in Tables 18 and 19, respectively, and those for female and male mice in the second experiment are shown in Tables 20 and 21, respectively. Small increases in the mean carbon chain length were observed when mice were fed the high fat diets. The increases observed when the mice were fed the polyunsaturated fat diet were due to the increased proportions

of linoleic and arachidonic acids, while those observed when the mice were fed the saturated fat diet were due to the increased proportion of oleic acid. Similar changes in mean chain length were observed in the second experiment.

In the first experiment the unsaturation index increased when mice were fed the polyunsaturated fat diet for 4 weeks. With continued feeding of the polyunsaturated fat diet the unsaturation index declined, but by week 30 the index was still higher than that when mice were fed the low fat diet. This was due to the increased proportion of oleic acid in plasma membrane lipids at week 30. The results of the second experiment confirmed that a transient increase in unsaturation of plasma membrane lipids occurred when mice were fed the polyunsaturated fat diet. In the first experiment the increased proportion of oleic acid in the plasma membrane lipids of mice fed the saturated fat diet was the main cause of the increased unsaturation of these membrane lipids at week 30. At this point, the plasma membrane lipids of mice fed the saturated fat diet were more unsaturated than those of mice fed the poly-unsaturated fat diet.

(b) Discussion

The major saturated fatty acids in the hepatocyte plasma membranes were palmitic and stearic acids while oleic, linoleic and arachidonic acids were the major unsaturated fatty acids. These fatty acids have been reported (van Hoeven and Emmelot, 1973) to be the major fatty acids in the phospholipids of mouse hepatocyte plasma membranes. The present observation of variation in the proportions of fatty acids in plasma

membranes isolated from different mice is supported by van Hoeven and Emmelot (1973) who showed that in mouse hepatocyte plasma membrane lipids large individual variation exists in the proportions of palmitic and stearic acids and that other minor fatty acids are not always present. It is also possible that the plasma membrane fractions contained variable amounts of impurities. These could have resulted from the isolation procedures which are much more difficult than those for other subcellular organelles (Bergelson, 1972). However, such problems are likely to be more important when comparing results from different laboratories and probably did not contribute significantly to the individual variation observed in the present experiments.

The small amount of arachidic acid in the plasma membrane lipids was probably derived from sphingomyelin (van Hoeven and Emmelot, 1973), which is present in significantly greater proportions in plasma membrane lipids than in the lipids of whole liver (Bergelson, 1972). The proportions of linoleic and arachidonic acids reported here were less than those reported for rat hepatocyte plasma membrane phospholipids by Keenan and Morré (1970). These workers found a greater proportion of linoleic acid in rat hepatocyte plasma membrane phosphatidylcholine than was observed in total lipids from mouse hepatocyte plasma membranes in the present experiments. However, Keenan and Morré (1970) also showed that sphingomyelin, a major plasma membrane phospholipid in these membranes, contained only a trace of linoleic acid and plasma membrane triglyceride contained only small proportions of

linoleic and arachidonic acids. This apparent difference in the fatty acid composition of hepatocyte plasma membrane lipids may therefore be due to the fact that, in the experiments reported here, the fatty acid composition of the total lipids extracted from the plasma membranes was determined. These results thus present the weighted mean proportions of fatty acids from all lipid classes.

It has now been conclusively demonstrated that increases in the proportion of fat in the diet lead to decreases in the *de novo* synthesis of fatty acids from acetate (Whitney and Roberts, 1955; Hill *et al.*, 1958, 1960; Bortz *et al.*, 1963) and in the activities of the two key enzymes involved, acetyl CoA carboxylase and fatty acid synthetase (Craig *et al.*, 1972; Liou and Donaldson, 1973; Wiegand *et al.*, 1973; Maragoudakis *et al.*, 1974). It is likely therefore that the feeding of the high fat diets to mice led to a suppression of the *de novo* synthesis of fatty acids. Thus, most of the fatty acid in the hepatocyte plasma membranes of the mice fed the high fat diets would have been derived from the dietary long chain fatty acids and not from acetyl CoA. The dietary fatty acids could have been incorporated unmodified or modified by elongation, desaturation, or both of these processes (Mead, 1960a, 1960b).

Using the EGSS-X, DEGS-PS and JXR gas liquid chromatography columns it was not possible to assign fatty acids to particular fatty acid families, such as the oleic ω9 or linolenic ω3 families. However, it is known that the linoleic and linolenic acids present in sunflower-seed oil, an ingredient

of the polyunsaturated fat diet, belong to the ω6 and ω3 families, respectively (Privett, 1968) and that inter-conversions of fatty acids from one family to another do not occur (Stumpf, 1969). Since linoleic acid (18:2 ω6) accounted for about 60% of all fatty acids in the polyunsaturated fat diet, it was probably the main precurser of the arachidonic acid present in increased proportions in the plasma membrane lipids of mice fed this diet. Linoleic acid could have been converted via γ-linolenic acid (18:3 ω6) or eicosadienoic acid (20:2 ω6) to arachidonic acid (20:4 ω6).

The hepatocyte plasma membranes of mice fed the saturated fat diet contained increased proportions of 18-carbon mono-unsaturated fatty acids. This may have resulted from increased incorporation of oleic or elaidic acids present in the dietary lipids. It was not possible to distinguish between oleic and elaidic acids with the gas chromatographic methods used, but tallow, an ingredient of the saturated fat diet, usually contains from 3 to 10% of elaidic acid (Shorland, 1955). This constitutes about a tenth to a quarter of the total 18 carbon mono-unsaturated acids. The increased proportion of 18-carbon mono-unsaturated fatty acids in the hepatocyte plasma membrane lipids could have also included oleic acid derived from desaturation of dietary stearic acid or elongation and desaturation of dietary palmitic acid. Herodek and Csakvary (1972) have reported that when rats are fed tallow, the proportion of oleic acid in the lipids of liver and adipose tissue increases above that in the diet, while the proportion of stearic acid increases only slightly.

It is interesting that the proportion of oleic acid in the hepatocyte plasma membrane lipids increased as mice were fed the saturated fat diet, whereas there was only a transient increase in the proportion of linoleic acid when mice were fed the polyunsaturated fat diet. Following this decline in the proportion of linoleic acid the proportion of oleic acid increased when mice were fed the polyunsaturated fat diet until week 30. This increase presumably resulted from incorporation of dietary oleic acid into the lipids of the hepatocyte plasma membranes. The biochemical explanation for the transient increase in the proportion of unsaturated fatty acids is not known. However, one could speculate that the increase in the amount of dietary linoleic acid overwhelmed systems designed to maintain a constant membrane structure.

The increased unsaturation and chain length of fatty acids of the plasma membrane lipids observed after mice had been fed the polyunsaturated fat diet indicate that the physical properties of the membranes may have changed. However, these changes, as monitored by changes in the phase transition temperature of the membrane lipids (Oldfield and Chapman, 1972), depend not only on the degree of unsaturation and chain length of the hydrocarbon chain, but also on the nature of the polar head groups of the lipid, the proportion of cholesterol in the membrane lipid and the degree of hydration. Thus the effect of the more unsaturated chains in lowering the transition temperature (Ladbrook *et al.*, 1968) would be antagonised to some extent by the effect of the increased hydrocarbon chain length (Chapman *et al.*, 1967). It is also possible that

decreased membrane hydration or substitution of phosphatidylcholine by phosphatidylethanolamine could have a similar effect (Stumpf, 1969) as these phospholipids usually have a different fatty acid composition. Studies on lipid classes isolated from mouse liver have shown that phosphatidylethanolamine has a higher proportion of stearic acid and a lower proportion of docosohexenoic and palmitic acids than phosphatidylcholine (Nelson, 1962). The cholesterol content of the membrane could also play a crucial role in modifying the physical properties of the membrane. In more unsaturated membranes cholesterol acts to strengthen the membrane, increasing its rigidity, and in more saturated membranes it acts as a membrane plasticiser or liquifier, increasing its fluidity (de Bernard, 1958; Shah and Schulman, 1967; Joos, 1970; Demel *et al.*, 1972).

From such theoretical considerations derived from data with model systems, it is interesting to examine published data on the effect of various lipid diets on the lipid composition and physical structure of natural membranes. Studies on the lipid composition of rat erythrocyte ghosts show that diets of different fatty acid composition produce very little change in the proportion of various lipid classes (Walker and Kummerow, 1963; de Gier and van Deenen, 1964) even though there are changes in the fatty acid composition (Witting *et al.*, 1961; Walker and Kummerow, 1963, 1964; Bloj *et al.*, 1973). The differences in the fatty acid composition and lipid distribution of erythrocyte ghosts from ruminants which hydrogenate dietary fat, and non-ruminants (de Gier and

van Deenen, 1961) are associated with differences in membrane permeability (Parpart and Dziemian, 1940). Changes in the degree of fatty acid unsaturation and cholesterol content of the membrane lipids of *Acholeplasma laidlawii* also lead to changes in the permeability and in the lipid phase transition temperature of the membranes (de Kruyff *et al.*, 1973a, 1973b). Thus it would appear that the changes in the degree of unsaturation of the fatty acids observed in the present experiments may have lead to changes in membrane physical properties such as permeability. As discussed in the Introduction, changes in the fatty acid composition of membranes also produce breaks in the Arrhenius plots of the activities of lipid-associated membrane-bound enzymes (de Kruyff *et al.*, 1973b). However, further studies on this topic should examine the diet-induced changes in the fatty acid composition of the different lipid classes as lipid-induced alterations in membrane function are thought to be determined mainly by the membrane phospholipids (van Deenen, 1965).

The results presented here are related to those of the experiments described in Sections 2 to 4 which showed that tumour incidences in DMBA-dosed animals and in mice inoculated with a tumour cell suspension were greater when the animals were fed the polyunsaturated fat diet rather than the saturated fat diet. Using the same dietary regimes as in the first experiment described in this section, the effect of the poly-unsaturated fat diet on DMBA-induced carcinogenesis was observed only when the diet was fed after administration of

the carcinogen. Other experiments have suggested that the diet fed in the first 4 weeks after dosing with the carcinogen is critical in influencing the tumour incidence (Carroll and Khor, 1975). Therefore, if changes in fatty acid composition, similar to those reported here, occurred in the tissues of DMBA-dosed and inoculated animals fed the polyunsaturated fat diet, one can speculate that the growth and proliferation of neoplastic cells in these animals was enhanced by higher concentrations of essential nutrients within the cells or by changes in the activity of various lipid-associated membrane-bound enzymes.

The finding of a transient increase in unsaturation of membrane lipids when mice were fed the polyunsaturated fat diet is also interesting in light of the suggestion, from the experiments with DMBA-dosed animals, that the change of diet may have been important in determining the tumour incidence. It is possible that a transient change in membrane structure, and perhaps other biological systems, could have favoured the proliferation of neoplastic cells in animals that commenced the polyunsaturated fat diet after receiving the DMBA.

These concepts are in accord with the demonstration of an association between membrane microviscosity and malignancy. The microviscosity in the hydrocarbon core of the plasma membrane lipid layer of normal lymphocytes has been shown to be almost 2 fold that of malignant lymphoma cells from mice (Inbar *et al.*, 1974; Shinitzky and Inbar, 1974). A similar difference was observed in human lymphocytes from normal

patients and chronic lymphocytic leukaemic patients (Inbar *et al.*, 1974). Increases in the membrane microviscosity of mouse lymphoma cells, achieved by manipulation of the proportion of cholesterol in the plasma membrane, were accompanied by decreases in the rate at which mice were killed by the tumour cells (Inbar and Shinitzky, 1974a, 1974b). In addition, Hui and Parsons (1976) have recently shown that the plasma membrane lipids of rat hepatoma cells have lower phase transition temperatures than those of normal rat hepatocytes.

SUMMARY AND CONCLUSIONS

Epidemiological data have implicated dietary fat as an important factor in the aetiology of various cancers in humans. This suggestion is supported by the results of experiments which have shown that animals fed high fat diets are more prone to develop certain types of tumours than are animals fed low fat diets. The experiments described in this thesis examined the influence of high fat diets containing either sunflower-seed oil (polyunsaturated fat diet) or tallow (saturated fat diet) on carcinogenesis. Changes induced by these diets in several biological systems involved in carcinogenesis were also studied.

1. The microsomal mixed function oxygenase system, which contains the haemoprotein cytochrome P-450, is responsible for the metabolism of many chemical carcinogens. As well as converting chemical carcinogens to their active electrophilic derivatives, these enzymes are responsible for the production of less carcinogenic derivatives. The balance between the activating and deactivating reactions is important in determining the effects of many carcinogens. The mixed function oxygenase enzymes are therefore important factors in chemical-induced carcinogenesis.

In the present experiments female rats were fed either the polyunsaturated fat diet or the saturated fat diet for 4 weeks. Rats fed the polyunsaturated fat diet slept for longer times after intraperitoneal injection of pentobarbitone than did rats fed the saturated fat diet. These results indicate a difference in the rate at which the drug was metabolised. Rats fed the polyunsaturated fat diet also had

lower concentrations of hepatic microsomal cytochrome P-450. The addition of pentobarbitone to the drinking water of rats caused 2 to 3 fold increases in the concentrations of hepatic microsomal cytochrome P-450, but did not alter relative effects of the diets.

A considerable number of experiments by other workers have shown that chemical carcinogenesis is inhibited by the induction of microsomal enzymes. The decreased microsomal metabolism of rats fed the polyunsaturated fat diet, compared to rats fed the saturated fat diet, may therefore have enhanced their susceptibility to the development of chemical-induced tumours.

2. The carcinogenic polycyclic hydrocarbon, 7,12-dimethylbenz(*a*)anthracene (DMBA) was used to examine the effect of different dietary fats on carcinogenesis in female rats. After receiving either the polyunsaturated or the saturated fat diet for 4 weeks, some of the rats received an intragastric dose of DMBA. In order to identify the stage of carcinogenesis which might be influenced by dietary fat, the diets of half of the rats were interchanged following DMBA administration so that rats previously fed the saturated fat diet were fed the polyunsaturated fat diet and vice-versa.

The cumulative incidence of tumour-bearing rats was greater among rats fed the polyunsaturated fat diet than among rats fed the saturated fat diet. The mean induction time of tumours was less and the proportion of tumour-bearing rats which developed malignant tumours was greater when rats

were fed the polyunsaturated fat diet. This enhancement of carcinogenesis was exerted only when the rats were fed the polyunsaturated fat diet after DMBA administration. It is therefore likely that this effect involved the promotional stage of carcinogenesis in which neoplastic cells proliferate to form a tumour. Since the initial event of neoplastic transformation appeared not to be involved, it is likely that the diet-induced changes in the mixed function oxygenase system, described in Section 1, were also not important in determining the tumorigenic response of the DMBA-dosed rats.

3. The effect of dietary fats on DMBA-induced carcinogenesis was examined using male and female C3HA^{vy}fB mice. Since the amount of DMBA required to induce tumours in these animals was not known, male and female mice fed a low fat diet received various amounts of DMBA in multiple and single intragastric doses. The multiple doses of DMBA tested were extremely toxic and resulted in the death of most of the mice. Increases in the amount of DMBA, given in a single dose, were accompanied by increases in the tumour incidence and decreases in the survival time of mice with or without tumours. The most common sites for tumours in male mice were the liver, lungs, skin and leukocyte-forming tissues. Most tumours in female mice occurred in the mammary glands and the ovaries.

The mice used to examine the influence of the dietary fats on carcinogenesis received a single intragastric dose of 5 mg DMBA and were fed the dietary regimes described in

Section 2. The results of the experiment with mice dosed with various amounts of DMBA and fed a low fat diet showed that 5 mg was a suitable amount of DMBA to induce tumours in these animals. The cumulative incidence of tumour-bearing female mice was greater among mice fed the polyunsaturated fat diet than among mice fed the saturated fat diet. As with the DMBA-dosed rats, this enhancement of carcinogenesis was observed only when the polyunsaturated fat diet was fed after DMBA administration. A similar enhancement occurred in male mice fed the polyunsaturated fat diet, but the number of males which developed tumours was relatively small and none of the differences between the tumour incidences were statistically significant.

The polyunsaturated fat diet appeared to be more effective in increasing the tumour incidence when the mice were fed the polyunsaturated fat diet for the first time after DMBA administration. This also occurred in the DMBA-dosed rats described in Section 2. It is possible that when animals commenced the diet the concentration in the tissues of the dietary factors enhancing carcinogenesis increased. Over a period of time homeostatic mechanisms may have partly restored the normal internal environment of the animals.

4. The results of the experiments with DMBA-dosed animals indicate that the enhancement of carcinogenesis by the polyunsaturated fat diet occurred during the promotional stage of carcinogenesis. It was therefore of interest to examine the effect of the diets on the proliferation of neoplastic cells to form discrete tumours. After receiving either

the polyunsaturated or the saturated fat diet for 4 weeks male and female mice were inoculated with a suspension of single cells from a transplantable mammary adenocarcinoma. The number of tumour cells in each inoculum was previously determined from an experiment which estimated the number of cells required to produce palpable tumours in 50% of inoculated mice fed the low fat diet.

The cumulative incidence of tumour-bearing mice was greater among both male and female mice fed the polyunsaturated fat diet, than among those fed the saturated fat diet. These results therefore support the hypothesis that the enhancement of carcinogenesis by the polyunsaturated fat diet is exerted during the promotional stage of carcinogenesis.

5. Since plasma membranes are involved in carcinogenesis, the diet-induced changes in the fatty acid composition of these membranes were studied using the same dietary regimes as in the experiments with DMBA-dosed animals. Hepatocyte plasma membranes were prepared from male and female mice using a combined rate and isopycnic zonal centrifugation technique. The effectiveness of this method in producing highly purified hepatocyte plasma membranes was checked by electron microscopy and by the assay of marker enzymes for the subcellular organelles.

There was some variation in the proportions of individual fatty acids in the hepatocyte plasma membrane lipids from different mice. Despite this variation, it was found that the compositions of the plasma membranes from male and female

mice fed the low fat diet were similar. The major fatty acids were palmitic and stearic acids while oleic, linoleic and arachidonic acids were the main unsaturated fatty acids.

The hepatocyte plasma membranes of mice fed the polyunsaturated fat diet for 4 weeks contained increased proportions of the major dietary fatty acid, linoleic acid, and increased proportions of arachidonic acid. The proportions of these fatty acids declined when mice continued to receive the polyunsaturated fat diet. The existence of this transient increase in unsaturation of the plasma membrane lipids of mice fed the polyunsaturated fat diet was confirmed in a second experiment. In this experiment, the proportions of linoleic and arachidonic acids reached a maximum after mice had been fed the polyunsaturated fat diet for 4 weeks, but declined after mice had received the diet for 8 weeks. The hepatocyte plasma membrane lipids of mice fed the saturated fat diet contained increased proportions of the main dietary unsaturated fatty acid, oleic acid. The increased proportion of oleic acid was the main cause for the increased unsaturation of the plasma membrane lipids of mice fed the saturated fat diet for 26 weeks. At this point, these membrane lipids were more unsaturated than those of mice fed the polyunsaturated fat diet for the same time.

The transient increase in unsaturation of plasma membrane lipids of mice fed the polyunsaturated fat diet could have been important in enhancing the development of tumours in animals which commenced the polyunsaturated fat diet after DMBA administration. The survival and proliferation of

neoplastic cells in these animals may have been enhanced by changes in membrane structure leading to altered membrane permeability or altered activities of lipid-associated membrane-bound enzymes.

In conclusion:

(a) Carcinogenesis was enhanced when DMBA-dosed rats and mice were fed a polyunsaturated fat diet rather than a saturated fat diet.

(b) The influence of the diets appeared to be exerted during the promotional stage of carcinogenesis which involves the proliferation of neoplastic cells to form a tumour.

(c) The increased tumour incidence observed in mice inoculated with a single cell suspension prepared from tumour tissue and fed a polyunsaturated fat diet, rather than a saturated fat diet, supported the above hypothesis.

(d) The observed diet-induced changes in the fatty acid composition of plasma membrane lipids indicated that such alterations could have contributed to the enhancement of carcinogenesis.

(e) Rats fed the saturated fat diet had greater rates of drug metabolism and concentrations of hepatic cytochrome P-450 than rats fed the polyunsaturated fat diet. These differences did not appear to be important in determining the tumorigenic response of the DMBA-dosed animals.

(f) Further investigation of the role of dietary fats in carcinogenesis is warranted. Topics which should be

studied include: (i) identification of the constituent(s) of the dietary fats responsible for the effect on carcinogenesis.

(ii) confirmation that the diet-induced enhancement of carcinogenesis is exerted on the survival and proliferation of neoplastic cells and not neoplastic transformation.

(iii) investigation of the actual mechanisms by which dietary polyunsaturated fat enhances carcinogenesis. These could include studies of diet-induced changes in the endocrine environment, in immune functions, in cell replication and in membrane function and synthesis.

APPENDIX

Computer programme for the identification of fatty acid methyl esters from gas liquid chromatography data

DATE 071376

```
      C      PROGRAM CHO45D                                                    000000
      C      ANALYSIS OF GAS CHROMATOGRAPHY DATA                               000000
             DIMENSION TTLE(13),NMAL(3,2),XMWEA(3),WTFA(50),HT(50),WD(50),WT(50 000000
            1),XMWFA(50),NDBIS(50),INDN(6),PM(50),PW(50),T(6),WTAL(3),WTALF(3), 000000
            2XX(3),YY(3)                                                        000000
             COMMON RT(50),INDX(50),NCAT(50),NA                                 000001
             IPL='+ FA'                                                         000001
             IFT='TTY'                                                          000003
             IAC='ACID'                                                         000005
             INT='RT.'                                                          000007
             IFIN='FIN'                                                         000011
             INUM='4500'                                                        000013
             IA='X'                                                             000015
             IN='N'                                                             000017
             IM='M'                                                             000021
             IBLNK=' '                                                          000023
             READ(5,101)ITST                                                    000025
         101 FORMAT(A3)                                                         000034
          57 IF(ITST-IFIN)1,2,1                                                 000034
           2 STOP                                                               000036
           1 IF(ITST-INUM)4,3,4                                                 000041
           4 WRITE(6,102)ITST,IFIN,INUM                                         000043
         102 FORMAT(///1CX,14HPROGRAM CHO45D,5X,31HA HEADER/TRAILER CARD CONTAI 000053
            1NS ,A3,1PH (AND NOT ,A3,4H OR ,A3,1PH ) IN COLS 1-3)               000053
             STOP                                                               000053
           3 READ(5,103)(TTLE(I),I=1,13)                                        000056
         103 FORMAT(13A6)                                                       000067
             WRITE(6,104)(TTLE(I),I=1,13)                                       000067
         104 FORMAT(1H1,10X,42HANALYSIS OF GAS LIQUID CHROMATOGRAPHY DATA,1CX,1 000101
            14HPROGRAM CHO45D,////  ,10X,13A6)                                 000101
             READ(5,105)XMWS,XMGMW,AMM,((NMAL(I,J),J=1,2),XMWEA(I),I=1,3)       000101
         105 FORMAT(3F8.0,3(A6,A2,F8.0))                                        000126
             SUMA=0.0                                                           000126
             SUMB=0.0                                                           000127
             NPT=0                                                              000130
             NX=0                                                               000131
             NN=0                                                               000132
             MARK=0                                                             000133
             RTMIN=100000.0                                                     000134
          24 NPT=NPT+1                                                          000137
             READ(5,110)ITST,NCAT(NPT),NDB,NISM,NSTAT,JX,N,P,RT(NPT),HT(NPT),WD 000141
            1(NPT),WT(NPT)                                                      000141
         110 FORMAT(A3,I2,3I1,3A1,4F8.0)                                        000164
             IF(ITST-IFIN)10,11,12                                              000164
          10 IF(ITST-INUM)12,11,12                                              000167
          12 XMWFA(NPT)=12.011*NCAT(NPT)+2.016*(NCAT(NPT)-NDB)+31.999+          000172
            1NSTAT*XMWS                                                         000172
             NDBIS(NPT)=    10*(10*NDB+NISM)+NSTAT                              000214
             IF(NN-5)333,13,13                                                  000222
         333 IF(N-IN)13,14,13                                                   000226
          14 NN=NN+1                                                            000231
             INDN(NN)=NPT                                                       000235
          13 IF(JX-IX)15,16,15                                                  000240
          16 NX=NX+1                                                            000242
             INDX(NX)=NPT                                                       000246
          15 PM(NPT)=HT(NPT)*WD(NPT)/(XMWFA(NPT)*XMGMW)                         000251
             IF(WT(NPT))17,18,17                                                000257
          17 PM(NPT)=PM(NPT)*WT(NPT)                                            000261
          18 PW(NPT)=PM(NPT)*XMWFA(NPT)                                         000264
             IF(M-IM)19,20,19                                                   000267
          20 MARK=NPT                                                           000272
             GOTO 21                                                            000274
          19 SUMA=SUMA+PM(NPT)                                                  000274
             SUMB=SUMB+PW(NPT)                                                  000301
          21 IF(RTMIN-ABS(RT(NPT)))24,24,23                                     000305
          23 RTMIN=ABS(RT(NPT))                                                 000311
             GOTO 24                                                            000313
      C       END OF CODING ON DATA INPUT                                       000313
          11 NPT=NPT-1                                                          000315
             IF(NX-2)25,881,881                                                 000317
         881 IF(RTMIN)801,801,802                                               000323
         801 WRITE(6,803)RTMIN                                                  000326
         803 FORMAT(//10X,7HNEG.OR ZERO RET.TIME - RTMIN= ,E11.4//)             000334
             GO TO 57                                                           000334
         802 IF(NX-3)95,26,26                                                   000336
          95 XKX=0.0                                                            000341
             SD=0.0                                                             000342
             CALL FNX(XKX,RSS,0,A,B)                                            000343
             GO TO 97                                                           000352
          26 XKX=0.0                                                            000354
             CALL FNMIN(XKX,0.1*RTMIN,0.00001,200,NCI)                          000354
             IF(2.0-NCI)27,27,28                                                000366
          27 WRITE(6,111)NCI,XKX                                                000372
         111 FORMAT(////10X,5HAFTER,I5,58H ITERATIONS THE MIN.RSS HAS NOT BEEN  000402
            1LOCATED - PRESENT K=,E11.4)                                        000402
          28 CALL FNX(XKX,RSS,0,A,B)                                            000402
             IF(RSS)201,202,202                                                 000410
         201 SD=0.0                                                             000413
             IF(RSS+0.0001*(NX-2))210,97,97                                     000414
         210 WRITE(6,109)RSS                                                    000425
         109 FORMAT(/9HRSS =/,E11.4)                                            000433
             GO TO 97                                                           000433
         202 SD=SQRT(RSS/(NX-2))                                                000435
          97 WRITE(6,112)A,B,XKX,SD                                             000450
         112 FORMAT(////10X,14HLOG(T*K)=A+B*C,//10X,22HWHERE T=RETENTION TIME,/ 000464
            116X,15HC=CARBON NUMBER,//10X,13HAND ESTIMATED,6X,2HA=,F9.4,/29X,2H 000464
            2B=,F9.4,/29X,2HK=,F9.4,//14X,17HS.D.(ABOUT LINE)=,F9.4)            000464
             WRITE(6,113)                                                       000464
         113 FORMAT(/10X,29H(LOGS ARE TO BASE E))                               000471
             WRITE(6,118)                                                       000471
             DO 32 I=1,NPT                                                      000502
             IF(RT(I))203,32,203                                                000502
         203 CRT=RT(I)+XKX                                                      000504
             TLOG=ALOG(CRT)                                                     000506
             FTLG=A+B*NCAT(I)                                                   000512
             ESTC=(TLOG-A)/B                                                    000520
             M=IBLNK                                                            000523
             IF(I-MARK)30,31,30                                                 000525
          31 M=IM                                                               000530
          30 JX=IX                                                              000533
             DO 34 J=1,NX                                                       000537
             IF(I-INDX(J))34,33,34                                              000537
          34 CONTINUE                                                           000544
             JX=IBLNK                                                           000544
```

```
      33 IF(NN)35,36,35                                                  000547
      35 DO 37 J=1,NN                                                    000550
         JJ=INDN(J)                                                      000555
      37 T(J)=CRT/(RT(JJ)+XKX)                                           000556
         IF(JX-IX)38,39,38                                               000565
      39 WRITE(6,120)NCAT(I),NDBIS(I),M,JX,RT(I),CRT,TLOG,FTLG,(T(J),J=1,NN  000570
     1)                                                                  000570
         GO TO 32                                                        000612
      38 WRITE(6,129)NCAT(I),NDBIS(I),M,JX,RT(I),CRT,TLOG,ESTC,(T(J),J=1,NN  000614
     1)                                                                  000614
         GO TO 32                                                        000640
      36 IF(JX-IX)138,139,138                                            000642
     139 WRITE(6,120)NCAT(I),NDBIS(I),M,JX,RT(I),CRT,TLOG,FTLG           000644
         GO TO 32                                                        000661
     138 WRITE(6,129)NCAT(I),NDBIS(I),M,JX,RT(I),CRT,TLOG,ESTC           000663
      32 CONTINUE                                                        000702
      25 IF(SUMA)57,57,804                                               000702
     804 WRITE(6,115)                                                    000704
         SWM=0.0                                                         000711
         SWA=0.0                                                         000712
         DO 50 I=1,NPT                                                   000720
         XMWME=XMWFA(I)*XMGMW                                            000720
         PMP=100.0*PM(I)/SUMA                                            000723
         PWP=100.0*PW(I)/SUMB                                            000727
         IF(MARK)52,51,52                                                000733
      52 IF(AAM)53,51,53                                                 000736
      53 WM=PM(I)*AWM/(PM(MARK)*XMWFA(MARK))                             000740
         WW=PW(I)*AAM/PW(MARK)                                           000745
         IF(I-MARK)204,55,204                                            000751
     204 SWM=SWM+WM                                                      000753
         SWA=SWW+WW                                                      000755
         M=IBLNK                                                         000757
         GO TO 54                                                        000761
      55 M=IM                                                            000763
      54 IF(WT(I))154,155,154                                            000765
     155 WT(I)=1.0                                                       000766
     154 WRITE(6,122)NCAT(I),NDBIS(I),M,HT(I),WD(I),WT(I),XMWME,XMWFA(I),  000771
     1PMP,M,PWP,M,WW,M                                                   000771
         WTFA(I)=WW                                                      001015
         GOTO 50                                                         001017
C         NO MARKER                                                     001017
      51 WRITE(6,121)NCAT(I),NDBIS(I),HT(I),WD(I),WT(I),XMWME,XMWFA(I),PMP,  001021
     1PWP                                                                001021
      50 CONTINUE                                                        001040
         IF(MARK)56,57,56                                                001040
      56 WRITE(6,192)SWM,SWW                                             001042
     192 FORMAT(/86X,F7.4,17X,F7.2)                                      001051
         IF(XMWEA(1))60,57,60                                            001051
C         PROCESSING FOR ESTERIFYING ALCOHOLS OPTION                    001051
      60 NAL=0                                                           001053
      63 NAL=NAL+1                                                       001055
         IF(XMWEA(NAL))61,805,61                                         001057
      61 WTAL(NAL)=0.0                                                   001062
         WTALF(NAL)=0.0                                                  001063
         IF(NAL-3)63,62,62                                               001064
     805 NAL=NAL-1                                                       001071
      62 WRITE(6,124)(IBLNK,IBLNK,IBLNK,I=1,NAL),(IWT,(NMAL(K,J),J=1,2),K=1  001074
     1,NAL)                                                              001074
         WRITE(6,125)(IWT,(NMAL(K,J),J=1,2),K=1,NAL),(IPL,IFT,IAC,I=1,NAL)  001130
         DO 64 I=1,NPT                                                   001163
         IF(MARK-I)65,64,65                                              001163
      65 DO 66 J=1,NAL                                                   001171
         XX(J)=WTFA(I)*XMWEA(J)/XMWFA(I)                                 001171
         YY(J)=XX(J)+WTFA(I)                                             001174
         WTAL(J)=WTAL(J)+XX(J)                                           001176
      66 WTALF(J)=WTALF(J)+YY(J)                                         001201
         WRITE(6,123)NCAT(I),NDBIS(I),WTFA(I),(XX(J),J=1,NAL),(YY(J),J=1,  001204
     1NAL)                                                               001204
      64 CONTINUE                                                        001231
         WRITE(6,126)SWA,(WTAL(J),J=1,NAL),(WTALF(J),J=1,NAL)            001231
         GO TO 57                                                        001251
     118 FORMAT(///15X,8HOBSERVED,3X,9HCORRECTED,/11X,2(3X,9HRETENTION),/9X  001254
     1,2(8X,4HTIME),6X,8HOBSERVED,5X,6HFITTED,4X,9HESTIMATED,5X,23HRETEN  001254
     2TION TIME RELATIVE,/5X,4HACID,8X,3H(T),8X,5H(T+K),5X,9HLOG (T+K),3  001254
     3X,9HLOG (T+K),3X,9HCARBON NO,5X,20HTO NAMED ACIDS .....,//)        001254
     115 FORMAT(///50X,9HMOLECULAR,15X,10HPERCENTAGE,14X,10HPERCENTAGE,/50X  001254
     1,9HWEIGHT OF,16X,8HOF FATTY,3X,9HWEIGHT OF,4X,8HOF FATTY,3X,9HWEIG  001254
     2HT OF,/50X,10HFATTY ACID,2X,9HMOLECULAR,4X,7HACID ON,4X,10HFATTY A  001254
     3CID,3X,7HACID ON,4X,10HFATTY ACID,/17X,4HPEAK,6X,4HPEAK,5X,9HWEIGH  001254
     4TING,5X,6HMETHYL,4X,9HWEIGHT OF,5X,5HMOLAR,6X,6HON MOLAR,5X,6HWEIGH  001254
     5HT,4X,9HON WEIGHT,/5X,4HACID,7X,6HHEIGHT,7X,5HWIDTH,4X,6HFACTOR,8X  001254
     6,5HESTER,5X,9HFREE ACID,5X,5HBASIS,3(7X,5HBASIS),//)               001254
     120 FORMAT(3X,I2,1H.,I3,1X,2A1,2X,F7.2,5X,F7.2,7X,F7.3,5X,F7.3,15X,5(2  001254
     1X,F7.3))                                                           001254
     129 FORMAT(3X,I2,1H.,I3,1X,2A1,2X,F7.2,5X,F7.2,7X,F7.3,17X,F7.3,3X,5(2  001254
     1X,F7.3))                                                           001254
     122 FORMAT(3X,I2,1H.,I3,1X,A1,3X,F7.2,5X,F7.2,4(5X,F7.2),1X,A1,3X,F7.4  001254
     1,1X,A1,2(3X,F7.2,1X,A1))                                           001254
     121 FORMAT(3X,I2,1H.,I3,6(5X,F7.2),17X,F7.2)                        001254
     123 FORMAT(3X,I2,'.',I3,2X,F9.4,6(5X,F9.4))                         001254
     124 FORMAT(////,14X,6HWEIGHT,2X,6(2X,3A4))                          001254
     125 FORMAT(4X,4HACID,4X,10HFATTY ACID,6(2X,3A4))                    001254
     126 FORMAT(/,6X,7(5X,F9.4))                                         001254
         END                                                             001254
```

```
      SUBROUTINE FNX(XK,RSS,NCD,A,B)                                     000002
      COMMON RT(50),INDX(50),NCAT(50),NX                                 000002
      SX=0.0                                                             000002
      CXX=0.0                                                            000005
      SY=0.0                                                             000006
      CYY=0.0                                                            000007
      CXY=0.0                                                            000010
      DO 1 I=1,NX                                                        000015
      IX=INDX(I)                                                         000015
      Y=ALOG(RT(IX)+XK)                                                  000017
      SX=SX+NCAT(IX)                                                     000026
    1 SY=SY+Y                                                            000033
      SX=SX/NX                                                           000036
      SY=SY/NX                                                           000040
      DO 4 I=1,NX                                                        000045
      IX=INDX(I)                                                         000045
      Y=ALOG(RT(IX)+XK)-SY                                               000047
      X=NCAT(IX)-SX                                                      000057
      CXX=CXX+X*X                                                        000064
      CYY=CYY+Y*Y                                                        000067
    4 CXY=CXY+X*Y                                                        000072
      RSS=CYY-CXY*CXY/CXX                                                000077
      IF(NCD)2,3,2                                                       000103
    3 B=CXY/CXX                                                          000105
      A=SY-B*SX                                                          000110
    2 RETURN                                                             000114
      END                                                                000146
```

```
      SUBROUTINE FNMIN(C,D,EPS,MAXNI,NCI)                               000000
      DIMENSION X(2),Y(2)                                               000000
      COMMON RI(50),INDX(50),NCAT(50),NX                                000000
      INTEGER SW                                                        000000
      X(1)=C                                                            000000
      X(2)=D                                                            000001
      NCI=0                                                             000003
      SW=0                                                              000004
      ILAST=0                                                           000005
      CALL FNX(X(1),Y(1),1,A,B)                                         000006
      CALL FNX(X(2),Y(2),1,A,B)                                         000015
      IP=1                                                              000024
120   IF(Y(1)-Y(2))5,5,6                                                000027
5     IP = 2                                                            000032
6     IF (ILAST - IP) 10,20,10                                          000035
10    ILAST = IP                                                        000037
      SW = 0                                                            000041
      GO TO 30                                                          000042
20    SW = 1                                                            000044
30    IF (SW) 40,40,50                                                  000046
40    F = 1.0                                                           000050
      GO TO 60                                                          000052
50    F = 0.5                                                           000054
60    IF (IP - 1) 70,70,80                                              000056
70    IOPP = 2                                                          000061
      GO TO 90                                                          000063
80    IOPP = 1                                                          000065
90    X(IP)=X(IOPP)+(X(IOPP)-X(IP))*F                                   000067
      CALL FNX(X(IP),Y(IP),1,A,B)                                       000075
      NCI = NCI + 1                                                     000110
      WX=ABS(X(IP))                                                     000113
      WX=ABS(Y(IP))                                                     000115
      IF(WX-0.1)201,202,202                                             000117
201   WX=0.1                                                            000122
202   IF(WY-0.0001)203,204,204                                          000125
203   WY=0.0001                                                         000130
204   IF(ABS(Y(1)-Y(2))-EPS*WY)101,101,110                              000133
101   IF(ABS(X(1)-X(2))-EPS*WX)100,100,120                              000142
110   IF  (MAXNI - NCI) 100,100,120                                     000154
100   C=0.5*(X(1)+X(2))                                                 000160
      D=X(2)                                                            000163
      RETURN                                                            000165
      END                                                               000222
```

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